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Kinetic study of ammonium/ammonia production by *Anabaena variabilis* cultures in relation with a continuous gas stripping

JURY

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Contents

Résumé

Contexte : des cyanobactéries diazotrophes pour réduire l'empreinte environnementale en microalgoculture

La production d'ingrédients pour les secteurs industriels associés à la pétrochimie à partir de ressources renouvelables est l'un des défis des sociétés contemporaines face à l'épuisement des réserves pétrolières. Les biomasses d'algues suscitent un intérêt croissant car elles peuvent être produites en photoautotrophie, donc impacter positivement le bilan carbone via la fixation photosynthétique du CO_2. Certaines microalgues font déjà l'objet d'applications industrielles, principalement dans les secteurs des ingrédients nutraceutiques, cosmétiques et agroalimentaire. Cette filière de production fait l'objet de nombreux travaux en vue d'une part d'optimiser la production des molécules énergétiques et d'autre part de réduire l'impact environnemental de cette production. La gestion de l'eau, donc son recyclage, et celle des nutriments nécessaires à ces productions font partie des principales contraintes. Dans ce contexte, l'utilisation de l'azote atmosphérique pour une bioproduction d'ammonium est une solution potentielle pour réduire le coût énergétique de la production de biomasse microalgale, la biofixation d'azote moléculaire étant, à priori, moins consommatrice d'énergie que la voie chimique par la réaction de Haber-Bosch.

Les cyanobactéries, ayant la capacité de fixer l'azote moléculaire, ont fait l'objet de nombreuses études pour identifier les réactions de la fixation d'azote (Wilson et al., 2012). Des résultats relativement récents ont démontré que la diazotrophie n'était pas une capacité réservée aux cyanobactéries multicellulaires, révélant ainsi des stratégies originales utilisées par ces microorganismes photosynthétiques pour assurer un processus qui requiert une anaérobiose stricte (Chen et al., 1996 ; Taniuchi et al., 2008). Les premiers travaux portant sur la photoproduction d'ammonium avaient pour objectifs des applications soit agricoles, particulièrement en riziculture, soit en chimie fine, particulièrement pour produire des phycobiliprotéines et des exopolysaccharides (Moreno et al., 1995 ; Moreno et al., 1998). Les souches sélectionnées, bien qu'aux propriétés potentiellement intéressantes, n'ont pas actuellement de réelles applications. Cet intérêt s'est accru avec les recherches menées en vue de leur utilisation pour produire des biocarburants tels que le méthane, les acides gras pour le biodiesel, le biohydrogène ou le bioéthanol. Ces biocarburants de troisième génération présentent l'avantage de ne pas solliciter l'usage de terres agricoles et donc d'interférer avec

les productions alimentaires. Toutefois, compte tenu des projections de production, les analyses de cycle de vie des voies de production ont mis en évidence la nécessité de trouver des solutions pour réduire leur impact environnemental. Ainsi certaines analyses suggèrent l'utilisation de cyanobactéries hétérocytées pour produire de l'ammonium extracellulaire en vue d'approvisionner en azote par voie biologique les cultures de masse de microalgues sans utiliser le chlorure d'ammonium, ou le nitrate de sodium dont la production par voie chimique est très énergivore, tout en favorisant le recyclage des eaux des récoltes de biomasse en évitant l'accumulation des ions chlorures ou sodium.

Objectifs de l'étude

Un travail précédent avait démontré qu'il est possible de provoquer une accumulation d'ammonium dans le milieu de culture d'une souche mutante de la cyanobactérie *Anabaena variabilis* en présence d'une concentration sub-inhibitrice de methionine-D, L-sulfoximine (MSX). Cette souche photoautotrophe a la propriété de pouvoir être cultivée en diazotrophie dans des photobioréacteurs avec des performances de croissance similaires à celles de la souche sauvage. Cette étude préliminaire avait permis d'établir l'importance de l'efficacité des transferts de gaz pour ce type de production, carbonatation pour le CO_2, azotation pour le N_2 et désoxygénation pour l'O_2. Ces données préliminaires ont permis d'améliorer les productivités en ammonium de cultures continues de la souche étudiée, mais aussi d'identifier certaines caractéristiques susceptibles d'impacter la faisabilité d'une bio-production potentielle d'engrais azoté.

Ainsi l'objectif global de la présente étude était d'améliorer les connaissances relatives aux cinétiques d'excrétion d'azote combiné résultant de la fixation d'azote moléculaire en relation avec des paramètres de conduite de cultures en photobioréacteurs. Les données bibliographiques ont permis de poser plusieurs hypothèses de travail, qui, de manière originale, prennent en compte la possibilité que l'excrétion de l'azote NH_4^+/NH_3 puisse modifier le pH des cultures, que ces modifications puissent avoir un impact sur un potentiel transfert de l'ammoniac en phase gazeuse et qu'un montage associant les cultures de la cyanobactérie à un barbotage de la phase gazeuse effluente dans une solution acide puisse permettre de mettre en évidence et quantifier un tel transfert gazeux.

Ainsi, le premier des objectifs était de fiabiliser et optimiser la production de biomasse de la souche, en vue d'en déterminer les performances en absence et en présence de

l'inhibiteur enzymatique, analogue structural du substrat. Le second objectif était de chercher à identifier les facteurs environnementaux favorisant l'excrétion de l'azote combiné et leurs impacts d'une part sur les échanges nutritionnels des cyanobactéries avec le milieu de culture et d'autre part sur des critères physiologiques des cellules cultivées en photoautotrophie. Enfin une analyse des données devait permettre de dimensionner un couplage potentiel de cette bioproduction d'azote combiné avec une culture de microalgue eucaryote, soit à comparer les cinétiques de production de la source azotée par le compartiment cyanobactérien avec celles de sa consommation par un compartiment microalgal.

Résultats et discussion

Nous avons confirmé que les propriétés de la souche mutante sont conservées plus de cinq ans après son isolement et son maintien par des réensemencements réguliers. Nous avons donc comparé les performances de croissance de la souche mutante à celle de la souche sauvage. En absence d'azote combiné, donc en diazotrophie, les cultures en mode batch des deux souches ont des taux de croissance spécifique du même ordre de grandeur. En culture continue dans des photobioréacteurs de 0.7 L, à un pH régulé à 6.8 par apport automatique de CO_2 et une température de 30 °C ces deux souches ont des valeurs de productivité en biomasse non significativement différentes, de l'ordre de 0.8 g L^{-1} j^{-1} pour un taux de dilution optimal de 2.5 j^{-1}.

Les valeurs du transport photosynthétique d'électrons global de la souche mutante sont significativement supérieures chez les cellules de la souche mutante aux conditions optimales de production de biomasse.

Cinétiques de production

Le processus cinétique de la production de NH_3 gazeux a été étudié de culture discontinue à la culture continue. En présence de MSX, l'azote fixé est excrété hors des cellules d'*A. variabilis* PCC 7937-C9 sous forme de NH_3. L'augmentation du pH des cultures des diazotrophes cyanobactéries peut être induite par l'ajout de MSX, selon la dissolution de NH_3 dans le milieu de culture. NH_3 peut être libéré à la phase gazeuse à pH élevé et pris au piège par la solution de HCl.

Nous montrons qu'une partie de l'azote excrété dans le milieu de culture est entrainée sous forme de NH_3 par la phase gazeuse, suggérant que les données publiées antérieurement

pourraient avoir été sous-estimées. Cette production dépend de la température, l'irradiance, le taux d'aération et la concentration en MSX. Des études cinétiques confirment que la production d'azote ammoniacal en phase liquide et en phase gazeuse est corrélée aux variations de pH. Une régulation pulsée de pH permet d'accroitre la production de NH_3. Des cultures en chemostat confirment que les productions de NH_3 gazeux sont maximales à pH 8.8. Une variation cyclique des teneurs en NH_4^+/NH_3 dissous semble réguler les teneurs en NH_4^+/NH_3 en dessous d'un seuil critique de 1.5 mmol L^{-1} via une consommation par les cellules végétatives. Ces caractéristiques physiologiques sont analysées pour une application potentielle à la fourniture d'azote à des cultures de microalgues oléagineuses.

Conclusion et perspectives

La culture en mode continu de la souche mutante C9 de la cyanobactérie *Anabaena variabilis* PCC 7937 a permis d'apporter des éléments de connaissance sur la physiologie de ce microorganisme photosynthétique en condition diazotrophique. Les régimes stationnaires obtenus grâce à ce mode de culture sont utilisés pour analyser de manière reproductible l'effet de différents paramètres sur :

- les cinétiques de production de biomasse,
- les relations entre croissance des cultures et consommation des nutriments
- les activités photosynthétiques en relation avec les variations de sa composition pigmentaire.

L'utilisation d'échantillons issus de ces cultures en mode continu a permis de mener des études correspondant à des incubations de courtes durées et ainsi de fiabiliser une étude physiologique visant à contrôler les conditions de production extracellulaire d'azote ammoniacal lorsque ces cyanobactéries sont traitées par un inhibiteur de la glutamine synthétase à des doses subléthales. Les données expérimentales obtenues répondent bien à l'objectif de l'étude, qui était d'améliorer les productivités en biomasse et en azote combiné par un microorganisme fixant l'azote moléculaire en condition de photosynthèse oxygénique.

Ainsi cette étude a permis de mettre en évidence :

- l'absence de différence significative entre la souche mutante C9 et la souche sauvage WT en termes de taux de croissance des cultures en chémostat ; toutes les deux

peuvent être cultivées à un taux de dilution de l'ordre de 3.0 d^{-1} sans lessivage de la culture.

- une meilleure efficacité photosynthétique de la souche mutante C9 par comparaison avec la souche type sauvage WT, révélée par des mesures de l'activité du PSII
- le transfert de NH_3 de la phase liquide à la phase gazeuse
- l'entrainement du NH_3 par la phase gazeuse (« stripping ») et son piégeage dans une solution de HCl rendant possible sa quantification en vue d'un bilan global de la production d'azote ammoniacal par les cyanobactéries
- une relation entre la production de NH_3 gazeux et l'accroissement du pH du milieu de culture; démontrée dans des cultures en mode discontinu pour atteindre une vitesse spécifique de production maximale de 620 µmol g X^{-1} d^{-1} dans la phase gazeuse. Le taux de production d'ammoniac dans la phase gazeuse dépend de la concentration en NH_4^+/NH_3 dans la phase liquide de la culture.

Plusieurs paramètres environnementaux impactant la production de NH_3 ont été identifiés grâce à plusieurs séries d'expérimentations, comme ;

- une concentration de 5 µmol L^{-1} de MSX est suffisante pour induire la production de NH_3 en phase gazeuse, bien qu'à 10 µmol L^{-1} la production en phase liquide soit significativement supérieure ;
- le taux d'aération des cultures, donc le coefficient volumétrique de transfert gazeux, avec un impact significatif sur l'excrétion de NH_4^+/NH_3 en phase liquide ainsi qu'en phase gazeuse ; ces données se réfèrent à l'importance du processus physique de transfert de gaz dans le processus de stripping mais aussi du processus de contrôle de la diffusion de l'azote combiné au travers de la membrane et de la paroi des hétérocytes de la cyanobactérie via le niveau d'accumulation de NH_4^+/NH_3 dans la milieu extracellulaire ;
- l'intensité des flux lumineux, avec une production maximale pour des PFDs de 100 µmol m^{-2} s^{-1} pour une concentration en biomasse de 0.30 g L^{-1} en masse sèche, des valeurs supérieures semblant photoinhibitrices pour la production de NH_4^+/NH_3;
- une température optimale de production de NH_4^+/NH_3 à 40 °C, soit 10°C de plus que la température appliquée lors de la production de la biomasse en culture continue ; les activités photosynthétiques sont également maximales à cette température ;

Des cultures en mode continu montrent qu'un niveau de 1.5 mmol L^{-1} NH_4^+ / NH_3 dans le milieu peut inhiber la production d'azote combiné dans le milieu extracellulaire ainsi que dans la phase gazeuse. Ainsi, il semble exister un seuil au-delà duquel l'azote libéré par les cyanobactéries provoque une inhibition de la fixation d'azote, les cellules le réassimilant ; cela se traduit par une diminution de la concentration de NH_4^+/ NH_3 dans la culture. Nous avons identifié que, dans nos conditions expérimentales, il semble bien exister un cycle d'accumulation/réassimilation, car en effet lorsque les valeurs des concentrations en NH_4^+/ NH_3 sont en dessous du seuil de quantification, un nouveau cycle d'accumulation/ réassimilation intervient. L'existence d'un tel cycle, qu'aucune étude préalable n'avait mis en évidence, est compatible avec les données bibliographiques relatives à l'impact des concentrations extracellulaires d'ammonium sur les activités nitrogénase.

Perspectives

Le modèle expérimental utilisé pour cette étude de faisabilité est une souche de cyanobactérie, sélectionnée pour sa résistance au MSX. Cette cyanobactérie filamenteuse, préalablement identifiée comme appartenant au genre *Nostoc*, ne produit de l'azote ammoniacal qu'en présence de doses subinhibitrices de cet analogue structural de la glutamine. Il serait intéressant de pouvoir remplacer ce modèle expérimental par :

- soit une souche excrétant naturellement de l'ammonium, des cyanobactéries symbiotiques du genre *Nostoc* pouvant présenter une telle propriété,
- soit une souche génétiquement modifiée pour réprimer partiellement l'activité glutamine synthétase.

La faisabilité d'un procédé de production continue d'ammonium/ammoniac par une telle souche implique également la maitrise du processus qui régule le cycle d'accumulation /réassimilation d'ammonium par la cyanobactérie. Il semble qu'une solution puisse être recherchée via une régulation dynamique du pH du milieu de culture intégrant les variations correspondante du rapport NH_4^+/NH_3. De manière non exclusive, une étude visant à maximiser les transferts gaz/liquide devrait permettre d'accroitre l'efficacité du processus d'entrainement de l'ammoniac par la phase gazeuse au sein du photobioréacteur.

Ce dernier point impliquera la conception d'une géométrie de photobioréacteur adapté pour une surproduction d'ammonium. Des essais préliminaires ont montré la possibilité de

conserver des cyanobactéries en diazotrophie à l'état immobilisé dans des billes d'alginate de calcium (Thèse L. A. Bui, 2013). Ce mode de culture est connu pour maximiser les teneurs en biomasse dans un bioréacteur, pouvant atteindre des valeurs de l'ordre de 30 g L^{-1} en régime permanent dans des cultures en mode perfusion. Le développement d'un tel procédé nécessitera la prise en compte de paramètres impactant la production d'azote combiné tels que ceux liés aux transferts de matière et d'énergie lumineuse au sein des gels utilisés. Une telle étude pourra nécessiter une adaptation de la géométrie des supports d'immobilisation et le mode d'agitation des bioréacteurs. Enfin, il existe d'autres moyens d'immobiliser des microorganismes pour séparer la production de biomasse de celle d'un produit excrété, en particulier ceux faisant appel à des technologies d'adsorption cellulaire.

Une des validations possibles d'un procédé de production d'azote ammoniacal par entrainement continu (strippage) de l'ammoniac par la phase gazeuse sera la mise en œuvre d'un couplage avec des cultures de microalgues assimilant cette forme d'azote minéral. Une modélisation des vitesses de transferts interphasiques et des vitesses d'excrétion d'un côté et des vitesses d'assimilation de l'autre côté sera nécessaire pour dimensionner un tel photobioréacteur à deux étages. Quoiqu'il en soit, les valeurs obtenues ont déjà permis une première simulation, qui montre que les valeurs obtenues dans l'état actuel de l'étude semblent compatibles pour une telle application. Des efforts de recherche restent encore nécessaires pour confirmer cette approche préliminaire.

General introduction

Background: N_2 fixation by heterocytous cyanobacteria for bio-fertilizer production

It is estimated that only about half of the current global population can be supplied with sufficient food energy and protein without the input of fertilizer nitrogen. The dependency on fertilizer inputs is increasing due to the anticipated increase in the population (Erisman *et al*., 2008; Gupta *et al.*, 2013). However, the Haber-Bosch chemical method used for industrial fertilizer production has high energy and CO_2 footprints. It has long been recognized that diazotrophic cyanobacteria have a great potential as bio-fertilizers as they are photoautotrophic microorganisms that require sunlight as a sole energy source for the fixation of carbon and nitrogen. Such an efficient process could decrease fuel demand for fertilizer production.

The cyanobacteria *Anabaena variabilis* Kützing *(A. variabilis)* is well known as one of the fast-growing heterocytous cyanobacteria for such purpose (Bui et al., 2014 ; Grizeau et al., 2015). When grown under photoautotrophic conditions, *A. variabilis* converts light energy (normally from the sun) into chemical energy which is stored in carbohydrate molecules, such as sugars, which are synthesized from CO_2 and water through photosynthesis process (**Equation 1**). This chemical energy can be subsequently released to fuel the organisms' activities.

$$6CO_2 + 6H_2O \xrightarrow{Photons} C_6H_{12}O_6 + 6O_2 \qquad \textbf{Equation 1}$$

Moreover, strains of this cyanobacterial group, such as *Anabaena sp.* PCC 7120, have been shown to assimilate organic, then could be cultivated in some wastewaters with both advantages to reduce the water footprint of its production and to participate to some circular economy through recycling effluent components into useful products (Yu et al., 2011).

These filamentous cyanobacteria have been extensively used as biological models for studying the beginnings of multicellular life. Indeed during their life cycle, their vegetative cells can differentiate into two cell types, including akinetes and heterocyts. Indeed under conditions of reactive nitrogen limitation, some filamentous cyanobacteria differentiate into heterocyts, whose sole function is N_2 fixation. They are formed by the transformation of about one every $10 - 20$ vegetative cells along the cell filaments (Lang et al., 1987; Muro-Pastor, 2014). In the absence of Nr sources, *A. variabilis* can use atmospheric N_2, which will be reduced to NH_3 by the highly O_2-sensitive enzyme nitrogenase (Nase). The reaction of N_2

fixation is described by **Equation 2**, it depends on electrons donated by ferredoxin, proton transport and ATP supply.

$$12H^+ + 12e^- + N_2 + 24MgATP \rightarrow 2NH_3 + 3H_2 + 24MgADP + 24P_i \quad \textbf{Equation 2}$$

Therefore, the heterocytous cyanobacteria strain *A. variabilis* could fix N_2 under photoautotrophic conditions. Indeed the vegetative cells have the capacity though their photosystem II for oxygen production from water splitting and the heterocyts have the capacity for dinitrogen fixation under extracellular oxygenic conditions, thanks to the low gas permeability of their cell-walls and to the absence of photosystem II.

Extracellular production of combined nitrogen in culture of diazotrophic cyanobacteria

There are several ways to produce extracellular combined nitrogen. The most frequently used but toxic way is to block the activity of glutamine synthase (GS) enzyme by adding L-Methionine-D, L-sulfoximine (MSX) (an inhibitor of GS). This chemical treatment prevents reaction between NH_3 and glutamate to form glutamine and as a result, NH_3 will accumulate before being released by the heterocyst (Bui et al., 2014 ; Grizeau et al., 2015).

When NH_3 is excreted of the cells dissolved into the culture, the following reaction will happen. The extracellular NH_3 will be transformed to be NH_4^+ in culture.

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \quad \textbf{Equilibrium 1}$$

The use of glutamate analogues, MSX, to induce NH_3 production by cyanobacteria, although technically feasible has some disadvantages. First, it is difficult to sustain prolonged NH_3 production in their presence in bioreactors, since negative impacts of MSX on the growth of cells; secondly, since MSX is toxic, it has to be separated from the extracellularly produced NH_4^+/NH_3 in culture prior to the use of the latter as a bio-fertilizer.

Then, to discover new strategy for using extracellular NH_3 produced as bio-fertilizer without toxic effect from *A. variabilis* cultures could be meaningful.

Objective

In previous study, a mutant cyanobacteria strain *A. variabilis* PCC 7937-C9 was obtained by double random mutagenesis treatments with EMS in our team (Bui et al., 2014). This strain has shown a long time tolerance to MSX without decreasing the capacity to produce extracellular NH_3. Our objective is to see if there could be a new strategy for using extracellular NH_3 as bio-fertilizer without toxic effect from this mutant *A. variabilis* cultures.

Hypothesis

As shown **Equilibrium 1**, the extracellular NH_3 will be transformed to be NH_4^+ in culture and change cultural pH, which has been observed in our previous study. The increase of pH could also lead to the equilibrium change of the distribution to which NH_3 forms NH_4^+, since it depends on the pH of the solution. Then, at high pH condition induced by MSX, there should be more extracellular NH_4^+/NH_3 in the form of NH_3.

Therefore, we establish the following hypothesis about the mechanism of gaseous NH_3 production from N_2 in *A. variabilis* culture. NH_3 is produced after N_2-fixation, and excreted out of cells in the form of NH_3 molecule when GS is inhibited by MSX (Böhme, 1998 ; Sah, 2008). Arriving in the culture medium, NH_3 dissolves in the medium in priority and transforms to NH_4^+ which causes pH increase. As more and more NH_4^+ appears in the medium, pH increases to a high level about 9.0, and NH_4^+ in the medium is at high level. Then, some of the NH_3 might be released to the gas phase and stripped by HCl solution from the gas outlet.

If there is gaseous NH_3 released into the gas phase, it will be much easier for the application of fixed-nitrogen as bio-fertilizer.

Organization of the thesis

Following this introduction, this Ph. D report is mainly organized according to five chapters.

Chapter I is a literature review, including the morphological, physiological characteristics of heterocytous cyanobacteria, and the mechanism and methods of extracellular NH_3 production by N_2-fixing cyanobacteria. Also, current strategy for extracellular NH_3 production and effect of environmental parameters on the growth of N_2-

fixing cyanobacteria are discussed. This bibliographic analysis has highlighted the need of a new strategy for using extracellular NH_3 as bio-fertilizer.

Chapter II is the presentation of the materials and methods. We described the equipments, their calibrations, and the detailed methods used for this study. This chapter includes a new designed setup for gaseous NH_3 stripping. The kinetic studies of gaseous NH_3 production in continuous cultures are particularly described.

Chapter III presents the comparison of the growth between *A. variabilis* mutant strain C9 and its wild-type, in order to characterize the mutant strain has the same growth potential as the wild type. For this purpose, the mutant strain C9 and its wild type strain WT were cultivated in an airlift photobioreactor. A series of growth rates at steady-state in continuous cultures from 0.3 d^{-1} to nearly 3.0 d^{-1} were obtained by setting feeding medium flow rates. Several steady states were achieved for both strains, no significant differences between C9 and WT except photosynthetic performance. The maximal specific growth rate without washing out of both reached about 3.0 division day^{-1}. The biomass productivity were, respectively, as high as 0.8 g L^{-1} d^{-1} at D=2.5 d^{-1} for C9 and 0.82 g L^{-1} d^{-1} at D=2.65 d^{-1} for WT, both of which were several times higher than ever studies. From DIC variation, it can be said that the cultures were still not saturated by CO_2, so higher biomass productivity could be obtained if more CO_2 were offered. Moreover, the mutant strain C9 showed better photosynthetic efficiency comparing to the wild type WT. This study is fundamental for further study in cyanobacteria, no matter for biomass application or development of their metabolites as high biomass productivity is basic.

Chapter IV presents kinetics of gaseous NH_3 excretion by *A. variabilis* mutant strain C9 in batch culture and the optimization of environmental factors for gaseous NH_3 production. As expected, NH_3 was detected in gas phase and successfully quantified by stripping in HCl solution. NH_3 liberation in batch culture occurred mainly at high pH (around 9.0) and maximal gaseous NH_3 productivity up to 1500 μmol g X^{-1} d^{-1}. pH pulse regulation with NaOH in batch culture could enhance NH_3 liberation to gas phase, which confirmed our hypothesis. Then, the effects of several environmental parameters for NH_3 production were optimized, such as temperature, irradiance, gas flow rate and MSX concentration on the excretion of NH_4^+ and NH_3. The results indicated that: 1) gas flow rate has significant impact on the excretion of both NH_4^+ and NH_3 that higher gas flow rate can obviously enhance the production of NH_4^+ and NH_3 ; 2) the best irradiance for the cells is 100 μmol photons m^{-2} s^{-1} at the cell concentration 0.30 g L^{-1} dry cell weight, too much irradiance was harmful to the cells and would decrease the NH_4^+/NH_3 production; 3) cells at 40 °C showed better

photosynthesis activities and more NH_4^+/NH_3 production than those at 20 and 30 °C; 4) no significant difference was observed among the tests with different MSX concentration at any condition. These observations will benefit the study and application of nitrogen fertilizers produced by cyanobacteria.

Chapter V deals with the kinetics of NH_3 excretion by *A. variabilis* mutant strain C9 in continuous cultures. In former studies, different factors affecting cell growth have been studied under batch culture, but very few reports can be found in continuous culture, particularly in chemostat. The aim of this study was to determine kinetics of biomass production, cell states and NH_4^+/NH_3 production in continuous culture. In order to further test of hypothesis of pH effect on gaseous NH_3 production, continuous cultures were conducted with pH continuous control. Continuous control pH successively at 6.6 and 8.8 in PBR showed that gaseous NH_3 production is pH dependent and no gaseous NH_3 produced at pH 6.6. Gaseous NH_3 production rate relates to the production of NH_4^+/NH_3 in culture medium. A level of 1.5 mmol L^{-1} NH_4^+/NH_3 in the culture could inhibit the Nase activity and hindered continuous gaseous NH_3 production. Then, cells could uptake the NH_4^+/NH_3 in the culture, leading to a decrease of NH_4^+/NH_3 concentration in the culture. However, Nase could reactivate and restart to biosynthesize NH_3 when NH_4^+/NH_3 in the culture concentration decreased to 0.

The aim of this thesis is to try to find a new strategy for using extracellular NH_3 as bio-fertilizer without toxic effect from cyanobacteria *A. variabilis* cultures. By theoretical analysis of the culture relating to pH, we discovered that it is possible to use the gaseous NH_3 from the *A. variabilis* mutant strain C9 cultures. The kinetics data from batch cultures and continuous cultures confirmed our hypothesis. Moreover, some of environmental factors on gaseous NH_3 are also optimized. From our general objectives, several perspectives to this work are envisaged. All these observations will benefit the study of N-biofertilizer and promote its application.

CHAPTER I Literature review

As it is known, nitrogen is, after hydrogen, carbon, and oxygen, the most abundant elemental constituent of all living organisms in the world. Among these, it is found particularly as a component of nucleic acids and proteins. Since it is needed for DNA and RNA, the molecules which store and transfer genetic information, nitrogen is essential for all living organisms. It is also required to make proteins, those indispensable structural components, receptors, and catalysts in all plant and animal cells. No matter humans, plants or microbes, their growth can't progress in absence of reactive nitrogen. In the nitrogen cycle, it exists in two forms divided as unreactive nitrogen (N_2) and reactive nitrogen (Nr) (Galloway et al., 2002). Among all the organisms, most are incapable to make use of N_2 which exist in chemically and biologically unusable gaseous form, but only Nr, such as ammonium, nitrate, glutamine, protein and so on, even though above 78 % of the earth's atmosphere is composed of N_2. In order to supply sufficient nitrogen nutrients for all the organisms, including a large population of humans, various types of plants and even microorganisms, industrial-scale fertilizer production has to be developed. So far, the most important method in use is dependent of the chemical reaction Haber-Bosch process.

The importance of Haber-Bosch process cannot be overestimated as billions of people have been fed. In the past 100 years, global population growth has accelerated markedly, from less than 2 billion to more than 7 billion (Erisman *et al.*, 2008). To feed all the humans, crops are indispensable in big amounts, and the crop yield should be largely increased at the same time. Due to an independent analysis, based on long-term experiments and national statistics, that about 30 − 50 % of the crop yield increase was due to the application of mineral N-fertilizer (Stewart *et al.*, 2005). By reacting atmospheric N_2 with H_2 to a chemically reactive and quite usable form of nitrogen (NH_3) at high temperatures under high pressures and in the presence of iron, Haber-Bosch process plays key role for supplying crops with reactive nitrogen nutrients during the past century (Erisman *et al.*, 2008). As a result, the number of humans supported by every hectare of arable land has increased from 1.9 to 4.3 persons between 1908 and 2008 thanks to Haber-Bosch nitrogen. It is estimated that only about half of the current global population can be supplied with sufficient food energy and protein if no input of N-fertilizer, and the anticipated increase in population to 2050 will increase the dependency on fertilizer inputs (Dawson and Hilton, 2011).

It is undeniable that Haber-Bosch process is benefiting humans on the earth, but it also leads to some problems, such as energy consumption and environment pollution. The energy used in Haber-Bosch process is equivalent to at least 32 MJ kg^{-1} Nr, or about 1.0 % of the global primary energy supply (Erisman et al., 2008). This could be considered as one of the key way of energy consumption all over the world. What's more, by looking at the present-day atmospheric and aquatic nitrogen pools, the influence of Haber-Bosch nitrogen on the global nitrogen cycle can also be seen. Since pre-industrial times, emissions of NH_3 and NO to the atmosphere have increased about fivefold (Galloway et al., 2004) . Besides, the average atmospheric nitrogen deposition rates have increased 20 times in large regions of the world, leading to unintentional fertilization and loss of terrestrial biodiversity. Similarly, the transfer of Nr from terrestrial to coastal systems has doubled since preindustrial times (Gruber and Galloway, 2008), as well as algal blooms and a quality decline of surface and ground waters. The intensive application of chemical fertilizers has substantially increased crop production, but there is an increasing concern over their adverse effects on long term soil fertility, soil productivity and environmental quality (Kannaiyan, 1993). Even though Haber-Bosch process is efficient for producing Nr, there are hundreds of millions of people who don't have enough food to eat caused by lack of sufficient Nr (Sanchez and Swaminathan, 2005). Thus, Haber-Bosch process has brought us many problems while feeding big part of humans. Are there some other methods to transfer unreactive nitrogen (N_2) to Nr in a low energy-consuming and more environmentally friendly way? If some other ways could help replenish the lack of Nr?

As it is known, N_2-fixing microorganisms are capable of transforming N_2 into Nr (inorganic compounds usable by plants). More than 90 % all N_2 fixation is realized by these organisms, which thus play an important role in nitrogen cycle (Dawson and Hilton, 2011).

Two kinds of N_2-fixing bacteria are recognized: free-living (non-symbiotic) bacteria, including the cyanobacteria (or blue-green algae) *Anabaena*, *Nostoc* and genera such as *Azotobacter*, and *Clostridium*, and mutualistic (symbiotic) bacteria such as *Rhizobium*, associated with leguminous plants (e.g., various members of the pea family), and certain *Azospirillum* species, associated with cereal grasses.

From the beginning of 19th century, cyanobacteria have attracted the attention of many researchers around the world thanks to their specific characteristic that certain species of them could fix atmospheric N_2 to a reactive form like ammonia (NH_4^+/NH_3), which is

potential to produce nitrogen fertilizers for agriculture and indirectly supply Nr for humans (Stewart, 1980).

1.1 The cyanobacterial world

As it is known, cyanobacteria (blue-green algae) are a critical component of the Earth's biosphere and are largely responsible for life, which are gram-negative oxygenic photosynthetic prokaryotes with a long evolutionary history. Before the Archaean and Proterozoic Eras (2.7 billion years ago), the atmosphere was unsuitable for life because of a very different chemistry composition from that at this moment (Kasting and Siefert, 2002). Cyanobacteria seem to be responsible for creating our O_2 atmosphere through photosynthetic activities, particularly through photosystem II (PSII) which split H_2O to release O_2. They were among the first organisms utilizing two photosystems (PSI and PSII) (Vermaas, 2001).

Cyanobacteria gets its common name "blue-green algae" from the blue-green pigment, phycocyanin, which along with chlorophyll *a* (Chl *a*) gives cyanobacteria a blue-green appearance. Even though both groups are photosynthetic and autotrophic, they are not nearly related because cyanobacteria are prokaryotic and lack internal organelles, a discrete nucleus and the histone proteins associated with eukaryotic chromosomes (Abed et al., 2009 ; Kumar et al., 2010). For long time, cyanobacteria have been considered as organisms between the eukaryotic and the prokaryotic according to their characteristics. Like the eukaryotic photosynthetic organisms, cyanobacteria can use light energy to fix carbons and produce biomass through PSI, while the cell walls of cyanobacteria contain peptidoglycan (as most bacteria) but not cellulose (as many plants and algae). What is interesting is that some cyanobacterial species are also diazotrophic, that is to say, they can survive via N_2-fixation in absence of reactive nitrogen (Nr). Being important contributors to global carbon and nitrogen budgets, cyanobacteria fulfill vital ecological functions in the world's oceans.

1.1.1 Classification of cyanobacteria

Many species of cyanobacteria have been identified till now. According to different index, they can be divided to different groups, as shown in **Figure 1−1**. For example, according to their appearance, they can be multicellular or unicellular. A brief introduction of their classification is now presented.

1.1.1.1 Free-living and symbiosis cyanobacteria

In light of cell structure and metabolites, cyanobacteria were likely part of a process of endosymbiosis by eukaryotic cells, resulting in the chloroplasts found in plant and algal cells. This living state is named symbiosis. According to the living states, cyanobacteria can be classified in two groups, free-living and in symbiosis (Meeks et al., 1985 ; Meeks and Elhai, 2002).

Free-living cyanobacteria are present in the water column in rice paddies, and can be found growing as epiphytes on the surfaces of green alga. For example, *Anabaena azollae*, a symbiont of the aquatic fern *Azolla*, can provide rice plantations with bio-fertilizer (Gupta et al., 2013).

The metabolites of free-living cells are altered when in association with other organisms. For example, the *in vitro* cultured filaments have been found to be morphologically different from fresh filaments from the fern cavity. Cells from the cavity are irregular in size and shape; those from *in vitro* cultures are very regular and generally smaller. Freshly obtained filaments have a higher heterocyt frequency than filaments from *in vitro* cultures (Gates et al., 1980).

N_2-fixing cyanobacteria form symbiosis with a wide variety of fungi, plants, and other organisms for benefiting them with Nr nutrients. In contrast to *Rhizobia*, cyanobacteria associate with representatives of all four of the major phylogenetic divisions of terrestrial plants: primitive spore-producing bryophytes (Villarreal and Renzaglia, 2006), ferns (Kannaiyan et al., 1994 ; Yadav et al., 2014) and the two classes of seed-producing plants, gymnosperms and angiosperms (Meeks and Elhai, 2002; Webber and Baker, 1996). Cyanobacteria could be found associated with corals, diatoms, dinoflagellates, seagrass and sponges (Wilkinson and Fay, 1979). By locating Nase both in free-living cyanobacteria *Anabaena* and in symbionts of *Cycas* and *Peltigera* (Bergman et al., 1986), it has been found that Nase only distribute throughout the heterocyt cytoplasma but not observed in the vegetative cells in both free-living *Anabaena* or in the cyanobionts, although the latter one apparently lives under microaerobic conditions. There are also many reports about cyanobacteria in free-living states and in symbiosis in recent years (Jackson et al., 2012 ; Picossi et al., 2013 ; Schouten et al., 2013).

It has also been found that Nase (Fe-protein) only distributed throughout the heterocyt cytoplasma in filamentous heterocytous cyanobacteria both in free-living state and in symbiosis, and that the Nase protein is also present in multiple heterocyts of cyanobiont (Bergman et al., 1986). In the marine cyanobacteria *Nostoc muscorum*, the same glycolipids have been found in freshwater representatives of these genera, but not detected in the *Hemiaulus-Richelia* association (Schouten et al., 2013). Instead, the glycolipids are identified to comprise a C_5 sugar, ribose, rather than C_6 sugars normally encountered in glycolipids of free-living cyanobacteria. The different glycolipid composition of the marine endosymbotic heterocytous cyanobacteria compared to their free-living counterparts is considered to be an adaptation to the high intracellular O_2 concentrations within their host.

1.1.1.2 Unicellular and multicellular cyanobacteria

Cyanobacteria are a large family of organisms with at least 3000 species. They exist in different morphologies, including unicellular and multicellular forms (including some in filament and some in colonies within a matrix) (Reddy et al., 1993 ; Zehr et al., 2001). Unicellular types exist as single cells, suspended or benthic, or aggregates, while filamentous types may be thin or thick, single trichome or bundles either with or without a sheath (Tease and Walker, 1987). The genera *Gloeothece* (Maryan et al., 1986 ; Tease and Walker, 1987), *Aphanothece* (Bastos et al., 2014 ; Waditee-Sirisattha et al., 2014), and Synechococcales (Zouni et al., 2001 ; Palenik et al., 2003) belong to unicellular forms (Steunou et al., 2006). The genera of multicellular cyanobacteria compose species in filament and species in colonies within a matrix. Chroococcales is an order of cyanobacteria which is characterized by single, floating cells or colonies embedded to a matrix (Reddy et al., 1993; Prasanna et al., 2006). Three orders of filamentous cyanobacteria are Oscillatoriales, Nostocales and Stigonematales. In Oscillatoriales, the cells are in uniseriate arrangement and do not form specialized cells (akinetes and heterocyts).

Unicellular cyanobacteria can also fix N_2 to NH_3, while carbon is also fixed by the same cell. With the cultures of *Gloeothece* sp. and *Synechococcus* sp., researchers have done some investigations on the mechanisms how they can manage both photosynthesis and N_2–fixation in the same cell (Mitsui et al., 1986). It has been found that N_2-fixation and photosynthesis occur at different phases in the cell division cycle. This observation is also proven by other studies that the doubling time of unicellular aerobic N_2–fixing cyanobacteria *Cyanothece* in presence of nitrate is 10 − 14 h and 16 − 20 h under N_2-fixing conditions

(Reddy et al., 1993). What's more, both studies found that the aerobic nitrogenase (Nase) activity is confined to the dark phase. Numerous inclusion granules formation has also been observed between the photosynthetic membranes in cells grown under N_2–fixing conditions.

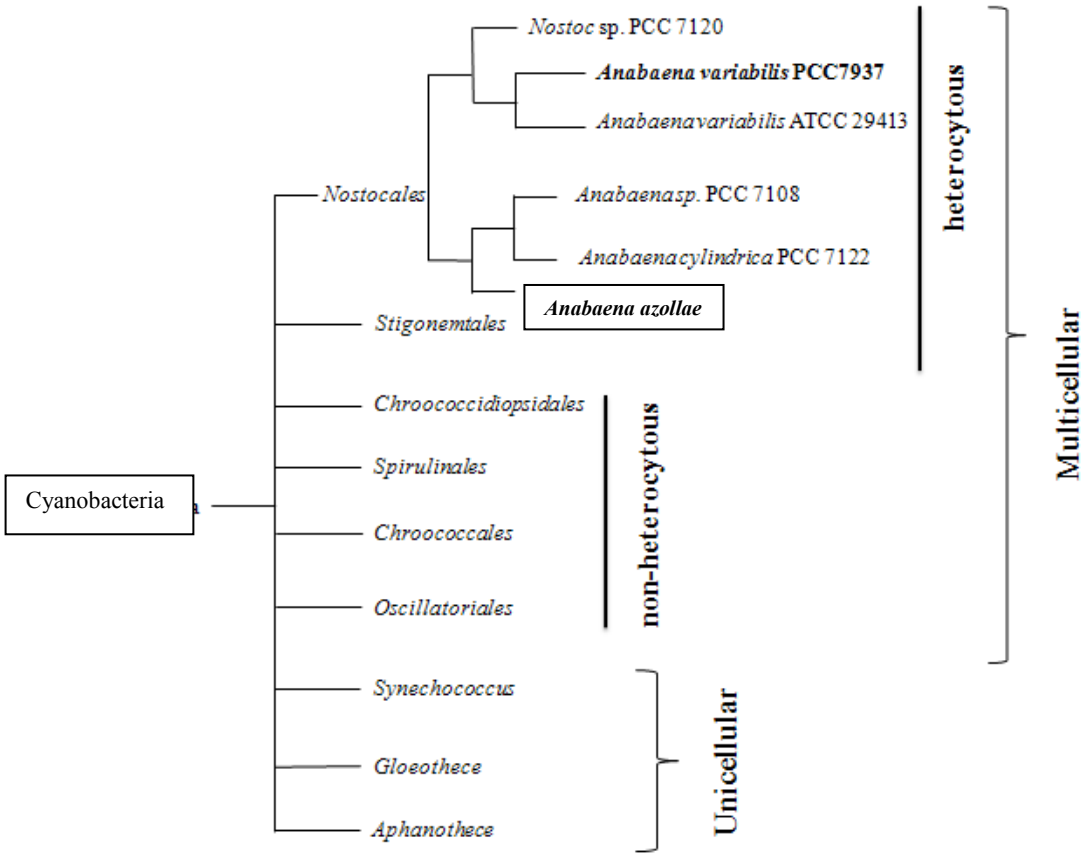


Figure 1−1. Phylogenetic tree of cyanobacteria based on 31 conserved protein sequences and 16S rRNA; adapted from (Davydov et al., 2013 ; Komárek et al., 2014 ; Tomitani et al., 2006 ; Zehr et al., 2007).

Under different conditions, filamentous cyanobacteria differentiate special cells. In general, they contain two kinds of cells, vegetative cells and specialized cells (heterocyts). With combined nitrogen in the growing medium, such as nitrate or ammonium, they grow as long filaments containing hundreds of photosynthetic vegetative cells. Nr deprivation is sufficient to induce heterocyt differentiation in all capable free-living N_2-fixing cyanobacteria. N_2-fixing cells "heterocyts" are differentiated terminally, forming at intervals of single heterocyt every ten to twenty vegetative cells along filament between stretches of vegetative cells to produce a multicellular pattern. Under light microscopy, heterocyts are distinguished from the vegetative cells by being colorless and apparently empty. As the sites of N_2-fixation, heterocyts have undergone various significant structural and biochemical changes which permit them to perform this activity. When habitat is in stressed conditions, cyanobacteria

evolve akinetes for survival, and hormogonia for dispersal and multiplication (Gupta et al., 2013). However, no single environmental factor has been identified that will induce hormogonium differentiation in all capable strains. A change in some environmental parameter can be the initiation of hormogonium differentiation, such as an increase or decrease of a nutrient or a change in the quantity or quality of light. Normally, vegetative cells fix carbons to produce organic compounds which will be transferred to heterocyts, in return, heterocyts supply amino acid and other forms of Nr for vegetative cells on basis of N_2– fixation (Fay, 1992; Walsby, 2007). The best studied filamentous cyanobacteria are primarily in the genus *Nostoc*, which includes many species of *Anabaena* strains.

Besides, the way of classification mentioned above, there are still some other ways to classify cyanobacteria. Concerning photosynthesis way, some of them are photoautotrophic, while some are photoheterotrophic. Also, some of them are toxic while others are not toxic. Anyhow, the ones which are more potential to be used as bio-fertilizers and most widely studied are the heterocytous photoautotrophic and diazotrophic strains. Then, we will have a detail introduction on the morphological characteristics of these strains.

1.1.2 Morphological characteristics of heterocytous cyanobacteria

As a model organism for studying the beginnings of multicellular life due to its filamentous characterization and cellular-differentiation capabilities, heterocytous cyanobacteria, such as *A. variabilis*, can differentiate three cell types during their life cycle, including vegetative cells, heterocyts and akinetes (**Figure 1−2**).

1.1.2.1 Vegetative cells

Cell Morphology

In the filaments of heterocytous cyanobacteria, vegetative cells are normally green or blue green, which are greener but narrower than heterocyts (**Figure 1−2**).

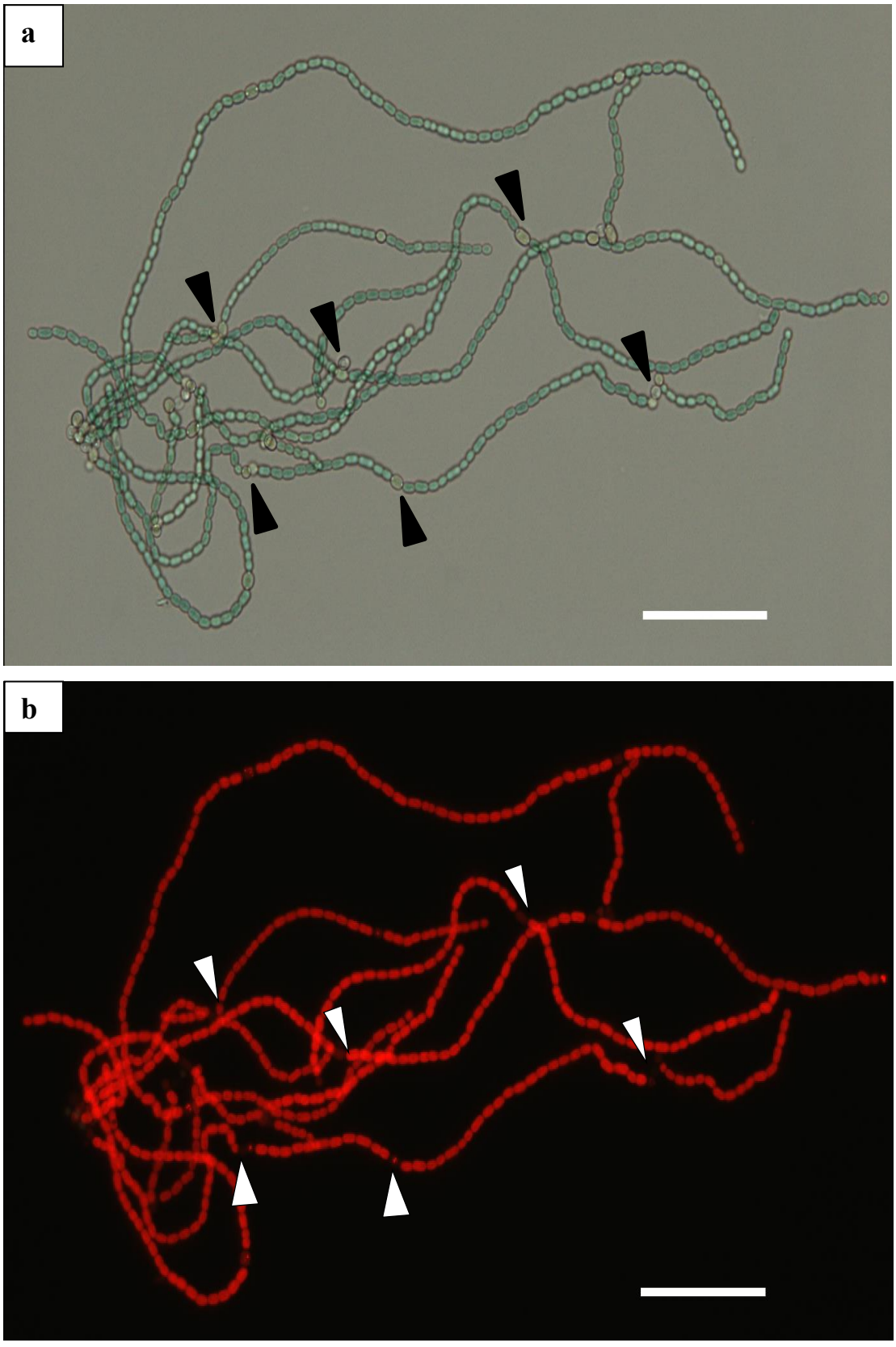


Figure 1−2. Microscopic observation of *A. variabilis* PCC7937-C9 under **(a)** light microscope and **(b)** fluorescence microscope ("Δ" refers to heterocyts; bar, 50 μm).

A study (Peat and Whitton, 1968) has reported the morphology and ultrastructure of *Anabaena* sp. Under different conditions, there are also some differences appearing in the cell

size and structure. Within 2 − 3 days of inoculation of an old culture into fresh medium, there appear filaments which show the narrowest width, least indentation and greatest mobility. At higher light intensities, for instance 6000 lux, cells are about 3.0 μm wide, while at lower light intensities they are somewhat narrower, the extreme forms is only 2.0 μm wide. In few cases of spore germination, filaments can be even narrower (1.5 − 1.8 μm). During cultivation, the mean width of the filaments and the degree of indentation at the cross-walls increases gradually, eventually the cell width reaches about 6.5 μm.

If the cells are growing well, long filaments appear to be with about 40 − 70 cells, but if the cells are under pressure, the filaments will be much shorter, with only 20 − 30 cells, or even only several cells. For example, wild type filaments grown in medium with 10 mmol L^{-1} $CaCl_2$, the filament size reduces considerably from 50 − 60 cells to 35 − 40 cells. As it is the case with wild type, the mutant strain also has showed extensive fragmentation in filaments. Reduction in the filament size is more pronounced under higher stress.

Cell Structure

Since cyanobacteria are prokaryotes, there is no cell nucleus, but the DNA suspends inside the cytoplasm, and no any organelle inside vegetative cells. Moreover, their walls bear a close structural resemblance to that of Gram–negative bacteria, which comprise a layer of peptidoglycan between the cytoplasmic membrane and an outer membrane, which can vary extensively in thickness among genera (Nicolaisen et al., 2009b). There is an opinion that the periplasmic space between the cytoplasmic and outer membranes might be continuous as the outer membrane is continuous along the filament observed with microscope (Flores et al., 2006).

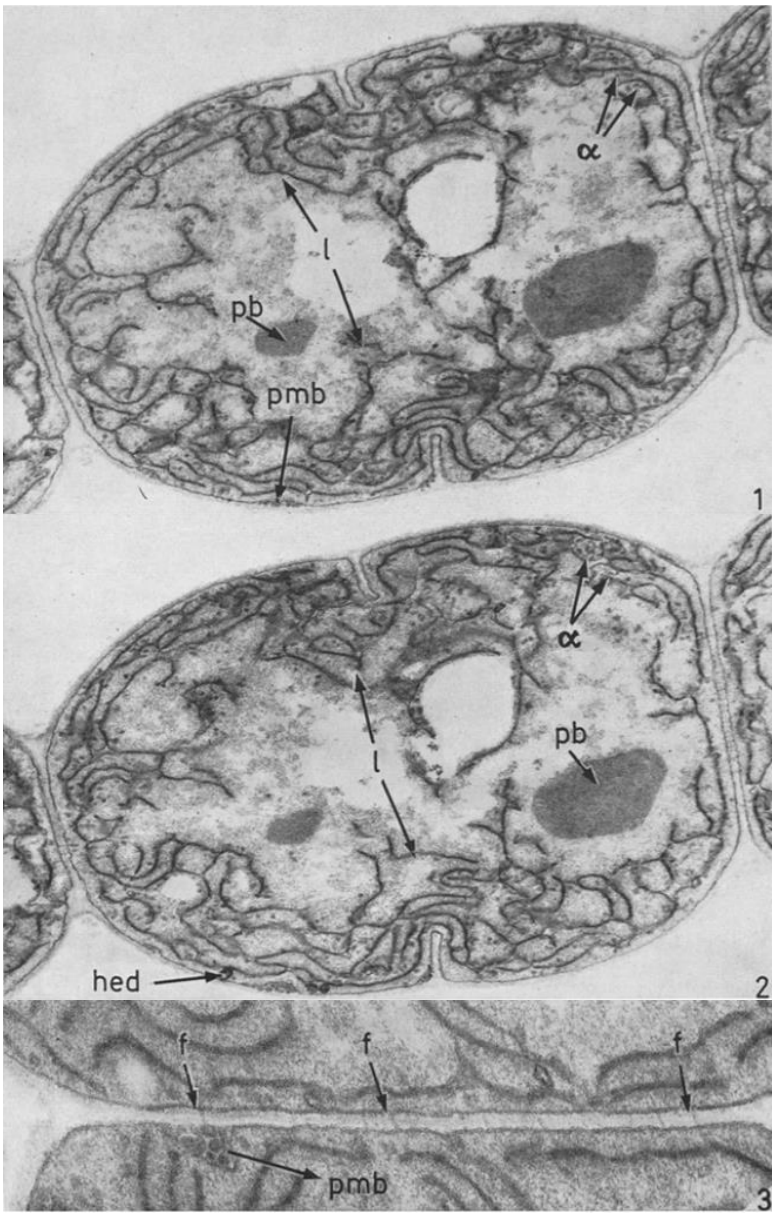


Figure 1−3. Structure of vegetative cells (1,2) adjacent serial sections through vegetative cell ×24,000, (3) section showing fibrils running between adjacent cells of a filament X75,000. (*α* = *α*-granule; *f* = fibril; *hed* = hollow electron dense body (possible β-granule); *l* = lamella; *pb* = polyhedral body; *pmb* = bodies bounded by plasma membrane) adapted from (Peat and Whitton, 1968).

However, there is big differences in the cell structure between heterocyts and vegetative cells. Vegetative cells are the normally growing cells of heterocytous cyanobacteria, in which the major part of carbon fixation occurs, inside which is full of thylakoid membranes (Kumazaki et al., 2013) and large cyanophycin granules accumulate (Lang and Fisher, 1969). With the help of three dimensional appearance of the cell, the lamellae form an anastomosing network (Peat and Whitton, 1968). Interesting structures in the organism include clusters of bodies about 30 μm in diameter which lie between the plasma membrane and the inner layer of the wall, and a developed system of fibrils running between

plasma membranes of adjacent cells across both the inner and middle wall layers. Vegetative cells of *Anabaena sp.* PCC 7120 and other cyanobacteria are surrounded by an inner and outer membrane, which are separated by a large periplasmic space and a peptidoglycan layer. External to the cell wall is a carbohydrate-enriched glycocalyx which can have different relative amounts of three recognizable layers: a closely associated sheath, a defined capsule, and loosely associated slime. These layers protect the cells from desiccation and presumably from phages and predators. Cyanobacteria contain extensive internal thylakoid membranes, the site of photosynthetic reactions (Kumar et al., 2010).

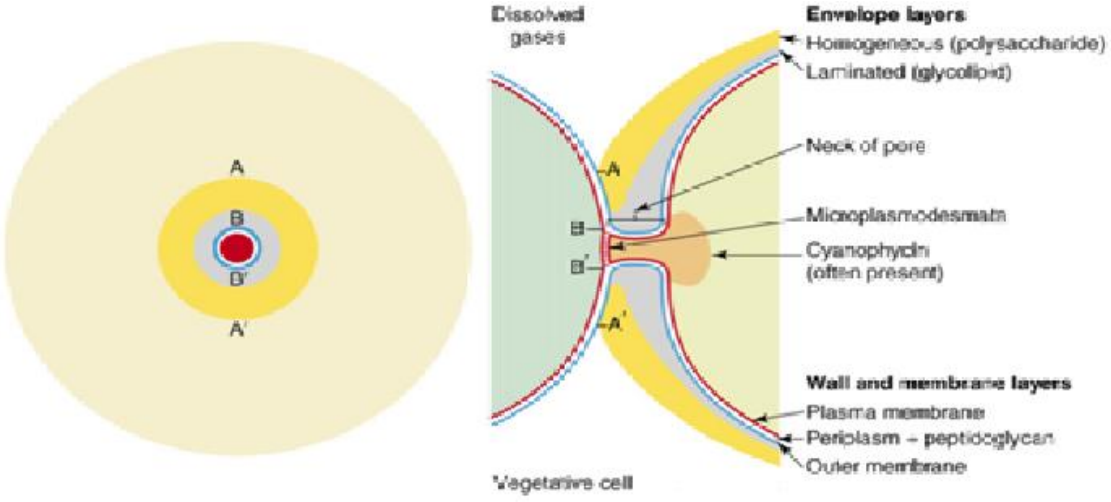
1.1.2.2 Heterocyts

Morphology

As is shown in **Figure 1−2** and **Figure 1−4**, heterocyts are typically distinguishable from vegetative cells by their somewhat larger and rounder shape, diminished pigmentation, thicker cell envelopes, and usually prominent cyanophycin granules at poles adjacent to vegetative cells; when seen under fluorescence microscope, they appear as spacing. In normal, heterocyts are round cells at larger diameter, or wider comparing to vegetative cells, which are always more than 4.5 μm wide (Peat and Whitton, 1968). In many *Anabaena* species, heterocyts are spaced at intervals of 10–20 cells along the filament, the arrangement can be explained by the growth pattern that all vegetative cells divide but not heterocyts. When the number of cells between two heterocyts rises to 20, one cell near the middle of the interval develops into a new heterocyt (Walsby, 2007).

Structure

As the specialized cells of *Anabaena*, heterocyts have been widely studied about their structure, particularly, the cell wall. These cells are characterized by a thick membrane that slows the diffusion of O_2, high PSI activity, the absence of PSII, and loss of division capability (Berman-Frank et al., 2003). The cell wall of heterocyts, differentiated from vegetative cells under Nr deprived conditions (Lehner et al., 2011), comprises three layers with other substances: inner glycolipidic layer, central polysaccharidic layer and outer fibrous layer. For details, the cell wall structure is shown in **Figure 1−4**.



End view a detached heterocyt **Longitudinal section through the heterocyt-cell junction**

Figure 1−4. The polar region of a heterocyt adjacent to vegetative cell; (adapted from Walsby, 2007).

In **Figure 1−4**, the detailed structure of heterocyt cell wall at the polar region adjacent to vegetative cell is shown. Clearly, the pore of a heterocyt comprises a narrow channel of cytoplasm, which often contains cyanophycin, and the bounding wall and membrane layers of plasma membrane, a periplasmic space with peptidoglycan sacculus and outer membrane. The structure is constricted by the thick layers of the heterocyt envelope, which contains two layers, including homogeneous polysaccharide layer and laminated glycolipid layer. In terms of the leakage of dissolved gas through the outer surface of heterocyt, N_2 and O_2 are proposed to enter the heterocyt through the pore via area *B, B'* but not by the envelope (Walsby, 1985). This speculation is opposite to the previous one that the main route for gas diffusion is through the envelope rather than through the terminal pores of the heterocyt (Kangatharalingam et al., 1992); and the rate of O_2 diffusion will limit the N_2-fixation rate in dark by limiting the rate at which ATP is supplied by oxidative phosphorylation.

Differentiation

It is well known that heterocyts are differentiated from vegetative cells by cell division. Heterocyt differentiates completely and irreversibly 12 − 20 h after Nr sources disappeared from culture medium (Berman-Frank et al., 2003). The whole process can be divided to three steps, namely, early proheterocyt (9 h), proheterocyt (12 h) and mature heterocyt (24 h), according to Nicolaisen et al. (Nicolaisen et al., 2009a). During the formation of heterocyts, the most dramatic morphological changes concern the cell wall. Besides, during the process,

the allophycocyanin (APC) content decreased till lost in mature heterocyts (Ke and Haselkorn, 2013). Recent studies have identified that histidine kinases, serine/threonine kinases and protein phosphatases have a more differentiated function in the regulation of heterocyt cell wall formation (Nicolaisen et al., 2009a).

During the last years, some genes associated to the development of heterocyts have been characterized. Nif2 gene is expressed long before heterocyt formation and shortly after Nr depletion, by which Nr can be supported when the filaments were independently of Nif1 Nase in the heterocyt (Thiel et al., 1997). In non–heterocytous species, Nif2 gene is also found in vegetative cells (Schiefer et al., 2002). Among them, the regulation by genes of NtcA and HetR are possibly involved in heterocyt development, furtherly Ca^{2+} release is considered to stimulate HetR activity (Nicolaisen et al., 2009a). Singh and Mishra have also proven that Ca^{2+} concentration has important influence on heterocyt frequency along the filaments (Singh and Mishra, 2014). The heterocyt frequency of *Anabaena* strains varies from 5.0 % to 10.0 % under diazotrophic conditions and restricts their N_2–fixing efficiency (Chaurasia and Apte, 2011).

Table 1−1
Comparison of cell composition between heterocyts and vegetative cells.

Cell composition	Heterocyts	Vegetative cells
Color	Pale yellow	Blue green
Thick firm cell envelope	Yes	No
Polar nodules	Yes	No
Internal thylakoid membrane	Less	More
PBP	Less	More
Photosystem	PSI	PSI and PSII
Normal cellular metabolism	No	Yes
N_2-fixation (Nase)	Yes	No
Cell division	No	Yes

Substance exchange between heterocyts and vegetative cells

Heterocyt–forming filamentous cyanobacteria are multicellular prokaryotes, in which heterocyts and vegetative cells have complementary metabolism and are mutually dependent. As a consequence of their specialization, heterocyts and vegetative cells have to be

metabolically interlinked because carbon fixation is limited to vegetative cells and N_2 fixation to heterocyts. It is largely agreed that on the one hand sucrose is a carbon source transferred from vegetative cells to heterocyts and on the other hand that glutamine is transported from heterocyts to vegetative cells for nitrogen supply (Thomas et al., 1977). This means that effective solute exchange mechanisms exist between these two cell types. In addition, the exchange mechanisms should also operate between vegetative cells because in *Anabaena sp.* PCC 7120 the ratio of heterocyts to vegetative cells is about 1 − 10 (Mullineaux et al., 2008). Different mechanisms or routes for the transfer of metabolites should exist.

Recent molecular and cellular studies have established the importance of molecular exchange between cells along the filament. Two current models propose either a continuous periplasm or direct connections between adjacent cells whose integrity requires the protein SepJ (Wilk et al., 2011). It is observed that each cell in the filament is individually surrounded by a plasma membrane and a peptidoglycan layer, and physical cell–cell contacts are mediated by intercellular connections, because the peptidoglycan layer of each cell enters the septum but the outer membrane does not.

The mechanism for metabolite exchange between cells has been investigated through studying the mechanism and kinetics of metabolite exchange of calcein in *Anabaena* (Mullineaux et al., 2008). From cytoplasm to cytoplasm, there are nonspecific intercellular channels allowing the movement of molecules. Exchange among vegetative cells becomes faster as filaments differentiate, which is considerably faster than exchange with heterocyts. Slower exchange is probably a price paid to maintain a micro–oxic environment in heterocyts, or is partially due to the presence of cyanophycin polar nodules of heterocyts (Breu et al., 2008 ; Mullineaux et al., 2008).

1.1.2.3 Akinetes

Morphology

Akinetes are thick-walled reproductive structures of cyanobacteria, which may serve as a resting stage resisting to adverse conditions and ensuring survival. Akinetes provide the capacity for germination after long-term exposure to stresses, such as cold, desiccation, and phosphate limitation.

Structure

The ultrastructure of akinetes is not very different from that of vegetative cells, but developed from vegetative cells along with the disappearance of several special organelles, for examples, gas vesicles. Nevertheless, there are some differences between akinetes and vegetative cells that they are normally larger than vegetative cells, with thicker cell walls, a multilayered extracellular envelope and contain large reserves of building materials such as carbon and nitrogen, such as glycogen granules, lipid bodies, and cyanophycin granules (Wan et al., 2011).

The most impressive difference between akinetes and vegetative cells concerns the cell wall, which is multilayered and sometimes decorated by various structures (warts, spines, special gelatinous and layered or radially structured envelopes). Akinetes and heterocyts of *A. variabilis* both form a conspicuous envelope, but they are distinguishable as follows (Zhou and Wolk, 2002). Whereas the pole of a heterocyt is perforated by a cytoplasmic channel which is surrounded by a thick layer of glycolipid, no such structure is found in akinetes. Often, akinetes are also more strongly pigmented and have a more granular interior.

Differentiation and function

Akinetes are spore-like nonmotile cells that differentiate from vegetative cells of filamentous cyanobacteria of the order Nostocales. They play a key role in the survival and distribution of this order and contribute to their perennial blooms. The major spark of akinetes differentiation is energy limitation according to Sutherland *et al.* (Sutherland et al., 1979). Various environmental factors are reported to trigger the differentiation of akinetes, including light intensity and quality, temperature, and nutrient deficiency. The principal requirement for akinete germination appears to be exposure to light (Kaplan-Levy et al., 2010). Akinete formation occurs within a restricted matrix of environmental conditions such as temperature, light intensity or photon flux.

Akinetes also could be induced by transferring the NO_3-grown culture to phosphate-free medium and incubation for two weeks under dim light (Lehner et al., 2013). Some studies have also reported that, in *Anabaena* strains, the differentiation of the first akinete is remote from heterocyts and young akinetes subsequently develop on either side of the first, and heterocyts may involve in akinete differentiation from vegetative cells (Fritsch et al., 1904 ; Kravchuk et al., 2011).

Akinete formation could be triggered by the deprivation of K^+ in culture medium of *Aphanizomenon ovalisporum*, which initiated 3 − 7 d after an induction by K^+ depletion, and followed by a maturation process (Sukenik et al., 2013). Phosphate and DNA replication are proven to be essential for akinete maturation. *Anabaena crassa*, however, produced akinetes upon potassium deficiency, but the highest akinete concentration is achieved at conditions that supported vegetative growth. It is speculated that an unknown internal signal is associated with the cellular response to K^+ deficiency to induce the differentiation of a certain vegetative cell in a trichome into an akinete and a universal stress protein that functions as mediator in K^+ deficiency signal transduction cascade, may communicate between the lack of K^+ and akinete induction (Sukenik et al., 2013).

In addition, researchers also did some investigation in order to suppress akinete germination as a potential management strategy for controlling cyanobacterial blooms. The conditions under which akinete germination of *A. circinalis* can be inhibited was investigated under laboratory conditions (Baker and Bellifemine, 2000). Results indicate that desiccation of akinetes for short term could significantly undermine their germinating ability.

1.1.3 Physiological characteristics of heterocytous cyanobacteria

Anabaena strains are among the very few groups which can perform non-oxygenic photosynthesis, respiration and N_2 fixation simultaneously in the same compartment. This combination of metabolic pathways is unusual, and this metabolic flexibility may be responsible for their evolutionary hardiness and their ability to thrive under a wide range of conditions. As is shown in last section, we have known that in the life cycle of *A. variabilis* there are three cell types, including vegetative cells, heterocyts and akinetes. Each of them performs distinct function. Vegetative cells are the basic cells for photosynthesis (depending on PSI/PSII) and respiration; heterocyts are mainly responsible for N_2–fixation, part of photosynthesis (only PSI) and respiration; akinetes are formed under stressed conditions for the continuation of the species like spores to fungi. Basing on the solid cell composition, the capabilities of *A. variabilis* attract our attention and the characteristics of photosynthesis and respiration will be introduced here.

1.1.3.1 Photosynthesis

Photosynthesis is a process used by plants, blue–green algae and other organisms to convert light energy (normally from the sun) into chemical energy which is stored in carbohydrate molecules, such as sugars, which are synthesized from CO_2 and water. This chemical energy can be subsequently released to fuel the organisms' activities.

Pigment composition

The pigments inside cyanobacteria, e.g. *A. variabilis*, include Chl *a*, total carotenoid, and phycobiliproteins (PBPs), including allophycocyanin (APC), phycocyanin (PC), and phycoerythrin (PE). The most striking characteristic of cyanobacteria is the presence of brilliantly colored accessory pigments, which contribute to the color blue-green, are the PBPs (PC, APC, and PE) (Silva et al., 1989). They are organized as supramolecular complexes, called phycobilisomes (PBS), which are attached in regular arrays to the thylakoid membranes. Excitation energy is transferred from PE through PC and APC onto the photosynthetic reaction center Chl *a*. PBPs may present at very high concentrations and may also serve as reserve proteins. They are water soluble and easily separate from the thylakoid membranes. The high levels of PBPs that cyanobacteria can accumulate under favorable environmental conditions (Kretz and Myers, 1955) make them attractive as a source of natural pigments for food products, such as beverages and dry beverage mixtures. These proteins are also widely used as fluorescent tags in a variety of diagnostic and research applications (Glazer and Stryer, 1984 ; Kronick, 1986).

It has been known that Chl *a* is whole for chlorophyll in most cyanobacteria, the content of which varies depending on species and cultivation conditions, even for the same strain the content could be from 3 to 14 mg L^{-1}. Researchers also separated heterocyts from the filaments of *Anabaena* strains and found that the Chl *a* content is almost equal between the vegetative cells and heterocyts but the C-PC content in heterocyts is about 50 % that of the vegetative cells (Kumar et al., 1982). Consistently, heterocyts of autotrophically grown *A. cylindrica* contain about 77 % as much Chl *a* as do vegetative cells, have little PC, and appear to lack myxoxanthophyll (Wolk and Simon, 1969). According to the quantitative data, individual vegetative cells contain an average of 0.386 μg Chl *a* per cell and isolated heterocyts have an average of 0.297 μg Chl *a* per cell, but they have much less PC than do vegetative cells. Light intensity has significant influence on the chlorophyll content (Lindblad

et al., 2002). The spectrum from purified akinetes resembles that from vegetative cells. The difference spectrum of methanol extracts of isolated hcterocyts and filaments shows a major difference in their carotenoids.

PSI, PSII

The organisms that perform photosynthesis are called photoautotrophs. Photosynthesis always begins when energy from light is absorbed by proteins that contain green chlorophyll pigments, in cyanobacteria they are embedded in the plasma membrane. The photosynthetic electron transport chain in cyanobacteria is essentially identical to that of plants, which consists of two photosystems, PSI and PSII. One important difference between cyanobacteria and plants is that the stoichiometry of PSI and PSII of cyanobacteria is much larger than 1.0, whereas an equal amount of PSI and PSII is the rule in higher plants (Scherer et al., 1988a).

Amongst, oxygenic photosynthesis via PSII (conversion of CO_2 and water to sugars using the energy from light) essentially is the reverse of respiration (conversion of sugars to CO_2 and water releasing energy). In PSII, some electrons are produced and then transported to PSI through electron carriers. In most cyanobacteria, photosynthetic electron transport occurs solely in thylakoids (Vermaas, 2001), whereas respiratory electron flow takes place in both the thylakoid and cytoplasmic membrane systems. As indicated, cyanobacteria utilize several redox active components in thylakoids for both photosynthesis and respiration. The soluble carrier of electrons could be plastocyanin (a copper containing enzyme) or cytochrome *c*553, relying on the species and on the availability of copper. Either of these soluble electron carriers can reduce the oxidized PSI reaction center chlorophyll.

Cyanobacteria harvest light energy across much of the visible spectrum using PBPs organized in cylindrical PBS, which transfer energy principally to PSII reaction centers. What's more, photosynthetic electron transfer leads to a proton gradient across the thylakoid membrane. In PSII, protons are released into the lumen upon water splitting, and protons formed upon plastoquinol oxidation are released into the lumen as well. The proton gradient across the thylakoid membrane is used for ATP synthesis by the ATP synthase in the thylakoid; this ATP may be applied for CO_2 fixation and for other cell processes.

According to the study (Kaplan et al., 1980), the apparent photosynthetic affinity of *A. variabilis* to CO_2 is greatly affected by CO_2 concentration in culture medium during growth. Half–maximal rate of photosynthetic O_2 evolution is achieved at 10 μmol L^{-1} and 100 μmol L^{-1} dissolved inorganic carbon (DIC) in cells grown at low–CO_2 (air) and high CO_2 (5.0% v/v CO_2 in air), respectively, whilst the maximum rate of photosynthesis is similar in both cases

(Kaplan et al., 1980). It has also been reported that cells growing under carbon sufficient conditions had photosynthetic affinities which are about 1000-fold lower than HCO_3^- limited cells (Miller et al., 1984).

Flavodiiron proteins have been proven to have crucial and specific roles in photoprotection of PSI and PSII in cyanobacteria (Ermakova et al., 2014). In *Anabaena* sp. strain PCC 7120, besides the four flavodiiron proteins Flv1A, Flv2, Flv3A, and Flv4 present in vegetative cells, there are two heterocyt–specific flavodiiron proteins, Flv1B and Flv3B which are employed for light induced O_2 uptake.

1.1.3.2 Respiration

In filamentous *Anabaena* strains, respiration appears in both vegetative cells and heterocyts, responsible for supplying ATP and reductants for cell metabolism, and for getting rid of O_2 in heterocyts for protecting Nase. The respiratory electron transport chain is apparently located both in the plasma membrane and within the thylakoid membranes. The cytoplasmic membrane, separating cytoplasm from periplasm, contains a respiratory electron transport chain but not photosynthetic complexes in most cyanobacteria. It is demonstrated (Ermakova et al., 2014) that Flv3B (one flavodiiron protein appears only in heterocyts) is responsible for light-induced O_2 uptake in heterocyts. It is further demonstrated that Flv3B-mediated photosynthetic O_2 uptake has a distinct role in heterocyts which cannot be substituted by respiratory O_2 uptake in the protection of Nase from oxidative damage and in an efficient provision of N_2 to filaments.

Function of respiration

Researchers have illustrated that respiration is the major source of reductants and ATP for Nase activity both in dark and in the light, but that PSI can contribute ATP at very high levels of illumination (Maryan et al., 1986). In consist, acetylene reduction activity (ARA) rate in *A. variabilis* in dark increased with increasing O_2 concentration, and a maximum value is reached when 30 % O_2 added in the gas phase, which presumably results from a requirement for energy provided by respiration (Jensen and Cox, 1983). No substantial difference in ATP level is observed between the light–inhibited and dark-kept cells (Imafuku and Katoh, 1976). Cellular respiration is also considered to play an important role during the transition, quenching the oxygen generated by photosynthesis and by providing energy necessary for the process (Bandyopadhyay et al., 2013).

Respiratory activities are influenced by light intensity and cell density

Respiratory activity, however, shows little response to the presence of external substrates (Vermaas, 2001). It's concluded that CO_2 evolved by respiration will be immediately refixed in the light without being liberated (Scherer and Böger, 1982).

Population density of non-diazotrophic cyanobacteria also influences the specific respiration rate, which appears to follow a similar inverse correlation at all population densities (Vonshak et al., 1982). This relationship is perhaps related to the decrease in O_2 pond caused by the increased population densities. The dark respiration rate of diazotrophically grown *A. variabilis* depends on O_2 concentration corresponding to the sum of high affinity and low affinity activities with Km values of 1.0 μmol L^{-1} and 69.0 μmol L^{-1} (Jensen and Cox, 1983). Only the high affinity activity is observed in nitrate-grown cyanobacteria lacking heterocyts. The O_2 concentration dependence of the low affinity activity resembled that for the stimulation of acetylene reduction. It is interpreted as the result of O_2 uptake by heterocyts, which is consistent with the idea that in intact filaments of cyanobacteria O_2 enters heterocyts much more slowly than it enters vegetative cells.

Photo–inhibition of respiration in cyanobacteria has been firstly reported in *Anabaena* sp. The remaining respiration in the light is observed to be at about 50 % of the rate in dark, even at low light intensity there is a clear inhibition of respiration (Ardelean and Peschek, 2011). As it is sustained, the light inhibition of respiration seems to be due to competition between electrons from the water splitting system and electrons from carbohydrate breakdown. The cellular localization of cytochrome *c* oxidase is demonstrated as a major factor controlling the degree of inhibition of respiration by light in cyanobacteria. Cell respiration within the heterocyts is a way for depleting O_2 from the intracellular medium. According to Imafuku and Katoh (1976), light preferentially absorbed by Chl *a* is responsible for the inhibition of respiration, but not that absorbed by the phycobiliproteins (Imafuku and Katoh, 1976).

1.1.3.3 N_2 fixation

N_2 fixation by cyanobacteria is catalyzed by nitrogenase (Nase) in the specialized cells, heterocyts, when cells are deprived of combined nitrogen Ammonia (NH_3) is the primary product of N_2 fixation which will normally be used by the cultures but won't be released out of the cells. Since in our study NH_3 excretion and production from N_2 fixation is the target, then it will be introduced in detail later.

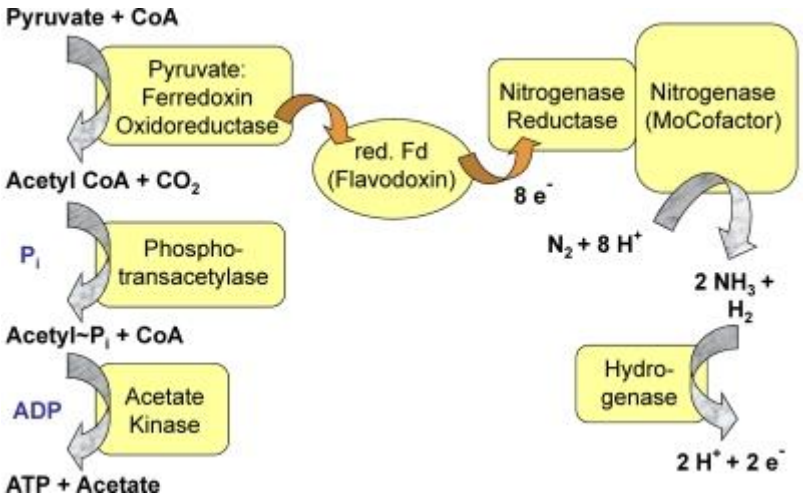


Figure 1−5. A simple scheme showing the relationship between pyruvate degradation, ammonium and hydrogen formation by nitrogenase, and hydrogen uptake by hydrogenase ; (adapted from Bothe et al., 2010)

1.1.4 Potential application of cyanobacteria

The overwhelming available knowledge on the diversity and physiology of cyanobacteria serves as an excellent base for exploring their applications in biotechnology. In the last few years, cyanobacteria have gained much attention as nutrient supplements in agriculture and industry, as food supplements and in wastewater treatment, for producing renewable energy H_2, and as a rich source of bioactive compounds.

Anabaena strains have several biotechnological applications as an excellent source of vitamins and proteins, fine chemicals and bioactive compounds, renewable fuel besides having bioremediation applications and as N_2–fixing bio–fertilizer (Wang et al., 1993).

1.1.4.1 Potential metabolites

Resources of pharmacy and food supplements

In the past, microalgae have been used in the diet of humans and animals mainly as supplements. In addition, cyanobacteria are known to produce wide array of bioactive compounds with diverse biological activities and chemical structures and have been considered as one of the most promising groups of organisms (Kreitlow et al., 1999 ; Nunnery et al., 2010). Investigations over last decades have identified compounds with for instance antiviral, antibacterial, antifungal, anticancer activity and plant growth regulators, having therapeutic, industrial and agricultural significance. Among all the secondary metabolites,

some are found to be directed against oxygenic photosynthetic processes and they are likely to be involved in the natural population regulation (Smith and Doan, 1999). Some cyanobacteria have also been found to intracellularly accumulate polyhydroxyalkanoates (Abed et al., 2009). From the review (Metting and Pyne, 1986), opinion is posed that secondary metabolite production by microalgae vary with environmental conditions and with better understanding of these processes and by optimizing and controlling the culture conditions, microalgae could become economic sources of new drugs and other specialty chemicals.

Toxins

Several cyanotoxins including microcystin, anatoxin, saxitoxin, cylindrospermopsin, and nodularin are known to be generated by a number of cyanobacteriae, which have now been shown to be produced by species of the planktonic genera *Anabaena*, *Aphanocapsa*, *Nostoc* and so on, even though not all strains of these organisms produce toxins (Vinogradova et al., 2011). Cyanobacterial toxins are bound to cells, but they will be released in water when cells die, is lysed or compromised. Blooms of freshwater cyanobacterium *A. circinalis* have been recognized to be an important health risk worldwide due to the production of a range of toxins, such as saxitoxin and its derivatives (Pomati et al., 2004). Among various saxitoxins, paralytic shellfish poisoning toxins have been identified to be the neurotoxins of *A. circinalis*. Some strains can also produce toxins which can be brushed off to skin and cause painful inflammation (Peschek et al., 2011).

Cylindrospermopsin (CYN) formed by the freshwater species of cyanobacteria species "*Aphanizomenon flos-aquae*" is of particular interest being marketed as a food supplement (Liu and Scott, 2011). However, through direct or indirect exposure, cyanobacteria as toxin producers also have disadvantages for humans and their environment. Cyanotoxin intake from contaminated water and food is an important source of human exposure. Various edible aquatic organisms, plants, and food supplements based on algae, can bioaccumulate these toxins and constitute a health risk for humans. So, the cyanobacterial dietary supplements must be properly evaluated in the perspective of safety (Liu and Scott, 2011; Vinogradova et al., 2011; Gutiérrez-Praena et al., 2013). Consequently, various detecting methods and techniques which can decrease the concentrations of cyanotoxins have been worked out.

1.1.4.2 H_2 production as renewable energy

H_2 produced by cyanobacteria is an attractive biofuel because it is made from water using sunlight as the energy source. In filamentous cyanobacteria, the primary enzyme used to produce H_2 is Nase and H_2 production takes place in heterocyts under aerobic conditions.

Studies have shown that H_2 production by *Anabaena* sp. is technically viable. Nr–starved cultures of *A. cylindrica* produced H_2 and O_2 continuously for 7 − 19 days, during which H_2 production attains a maximum level after 1 − 2 days of starvation followed by a slow decline (Weissman and Benemann, 1977). The addition of 0.1 − 0.5 μmol L^{-1} NH_4^+ increased total H_2 production, in contrast, 0.2 mmol L^{-1} NH_4^+ addition at various times destabilized the system and eventually suppressed H_2 production completely (Jeffries et al., 1978). Possible factors which limit H_2 production may be the decline of reductant supply and filament breakage.

For *Anabaena* PCC 7120, the rate of H_2 production increases with light in a photobioreactor (PBR) using air as the lifting gas. H_2 production increases significantly when replacing the air with argon. Despite relatively high production, maximal efficiency of solar energy to H_2 conversion is only 0.042 % (Lindblad et al., 2002). Study in production of H_2 by *A. cylindrica* sparged with argon gas in the absence of exogenous Nr has shown that the production can continue for 30 days under limited light conditions and for 18 days under elevated light conditions (Jeffries et al., 1978). In consistence, H_2 production by Nase is proven to be independent upon the reduction of N_2 and Nase produces only H_2 in an argon atmosphere (Weyman et al., 2010).

1.1.4.3 Wastewater treatment for environment protection

An emerging area of interest in cyanobacteria application is wastewater treatment (Lincoln et al., 1996 ; Christenson and Sims, 2011), which has been proven to be cost–effective and beneficial. Pouliot *et al.* studied about the effects of different factors which influence the treatment efficiency of wastewater by cyanobacteria cultures and found that the best aeration–agitation mode is aerating the culture with bubbles on a 14/24h light dark cycle while stirring bars appear to be an unsuitable system for filamentous cyanobacteria (Pouliot et al., 1989). Laboratory experiments conducted on effluent from an anaerobic lagoon of a modem dairy show that cyanobacteria grow well on dairy wastewater and that nitrogen can be removed rapidly and completely. NH_3 nitrogen concentrations are significantly reduced in 7 days during which the maximum removal rate reached 24 mg L^{-1} d^{-1} (Lincoln et al., 1996).

Some other researchers also tested the potential of high latitude cyanobacteria for tertiary wastewater treatment by analyzing the removal rates of nitrogen and phosphorus indicating that even polar cyanobacteria from extreme cold conditions cannot work well on wastewater treatment at low temperature (Chevalier et al., 2000). Strain *A. doliolum* has also been confirmed as an agent for low cost biological treatment of domestic wastewater through nutrient manipulation (Dash and Pradhan, 2013).

1.1.4.4 NH_3 production as bio–fertilizer supplier

As mentioned above, cyanobacteria can use sunlight as the sole energy source for the fixation of carbon and nitrogen, which donates them the potential of being applied in agriculture and industry, as nutrient supplements, such as bio-fertilizer which is supposed to be a safe alternative to chemical fertilizers to minimize the ecological disturbance. Bio–fertilizer is a substance containing living microorganisms when applied to seed and plant surface, colonized the rhizosphere or the interior part of the plant and promoting growth by increasing the availability of primary nutrient to the host plants.

NH_3 is the initial product of N_2 fixation by pure cultures of *Anabaena* sp., and glutamine and glutamate are the primary organic products synthesized from N_2. Some N_2-fixing cyanobacteria, such as *Anabaena* strains, are found to release varying quantities of NH_3 without any induction, both in the presence and absence of nitrate in the medium, during different phases of their growth. In general, growth and NH_3 release are comparable in both media, although there are strain differences. Three patterns of NH_3 release observed in different strains during their growth period appear to be: 1) release pattern parallel to the growth curve, 2) a continuous increase in release and 3) release showing a bimodal curve (Subramanian and Shanmugasundaram, 1986). Several researchers have also shown that cyanobacteriae can excrete a portion of fixed nitrogen into the medium in the form of amino acids and peptides. According to Fogg (Fogg, 1949), as much as 30 % − 40 % of the fixed nitrogen by *A. cylindrica* appears in the medium in soluble form. However, in other study, it is also shown that most of the fixed nitrogen remains in the cells, no more than 7.0 % – 8.0 % appearing in the medium. *Anabaena,* immediately isolated from *Azolla*, releases about 50 % of its fixed nitrogen as NH_4^+ (Peters, 1977). The released NH_4^+ could be assimilated by *Azolla* through either the glutamine synthetase-glutamate synthase (GS-GOGAT) or glutamate dehydrogenase (GDH) pathways (Ray et al., 1978). *A. variabilis* is able to release extracellular NH_3 to a concentration of 27.5 μg mL^{-1} (Tiwari et al., 2010). The assimilation of

exogenous NH_3 into glutamine and glutamate by isolated symbiotic *Nostoc* can be inhibited by L–methionine–D, L–sulfoximine (MSX), and consequently less than 1.0 % of fixed nitrogen is lost to the suspension medium (Meeks et al., 1985).

After five years of study, results have been obtained that the rice yield is increased by using cyanobacteria as bio-fertilizer, in which the gain yield and straw yield are increased by 7 % − 20 % (Paudel et al., 2012). Studies for testing the potentiality of wild type and mutant strains of *A. variabilis* to colonize with the roots of rice and wheat plants has been undertaken by Datta et al., they found that all the strains are able to colonize the roots of rice and wheat, and the N_2 fixation rates and Chl *a* content of the associated strains are higher than those in free-living cells (Datta et al., 2014). All these reports have illustrated the potential of cyanobacteria as bio-fertilizer for raising crop production.

1.2 Mechanism and methods of extracellular NH_3 production by N_2-fixing cyanobacteria

1.2.1 *In vivo* reaction of N_2 fixation

Under conditions of Nr limitation, many filamentous cyanobacteria differentiate cells called heterocyts, whose sole function is N_2 fixation. They are formed by the transformation of about every 10 − 20 vegetative cells and are more or less evenly distributed along the filaments (Lang et al., 1987 ; Spiller et al., 1986). In the absence of Nr sources, specialized strains can use atmospheric N_2, which will be reduced to NH_3 by the highly O_2–sensitive enzyme Nase. Various strategies are employed by different strains to avoid O_2 poisoning of Nase.

$$12H^+ + 12e^- + N_2 + 24\text{MgATP} \rightarrow 2NH_3 + 3H_2 + 24\text{MgADP} + 24P_i \quad \textbf{Eq. 1−1}$$

The reaction of N_2 fixation is shown as above, from which it's easy to know that N_2 fixation bases on electron transport, proton transport and ATP supply. Details of the metabolism occurring in heterocyt are as shown in **Figure 1–5**.

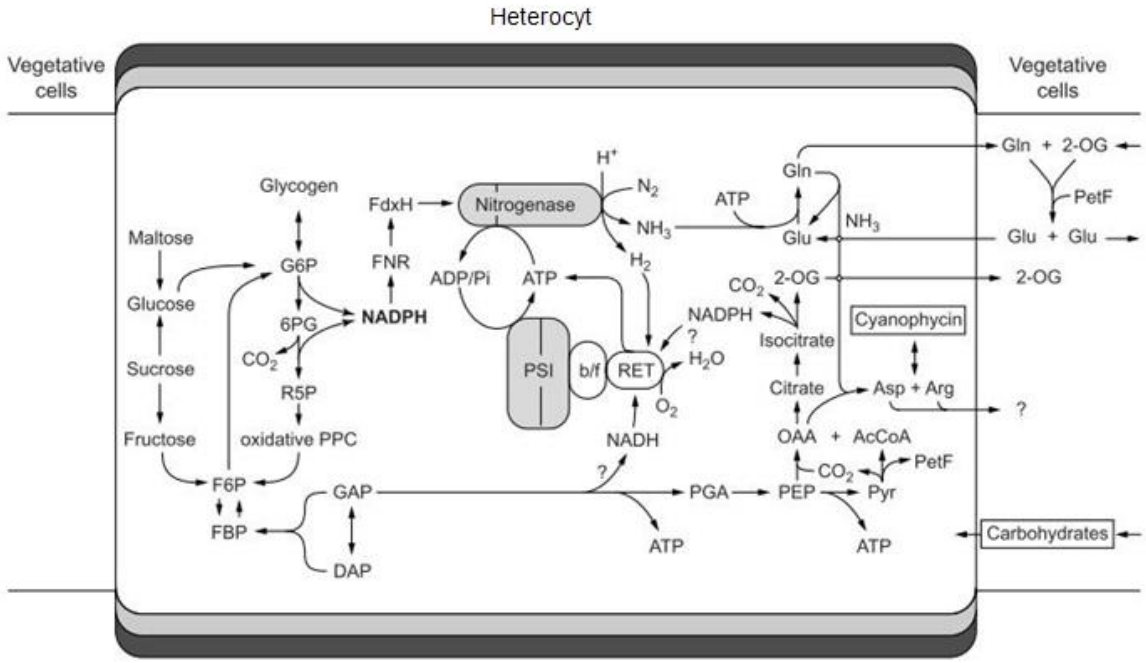


Figure 1−6. Heterocyt metabolism and N_2 fixation.
AcCoA = acetyl coenzyme A; Arg = arginine; Asp = aspartate; b/f = cytochrome b6f complex; F6P = fructose 6–phosphate; PetF = vegetative cell type ferredoxin; Glu = glutamate; Gln = glutamine; OAA = oxaloacetate; 2–OG = 2–oxoglutarate; 6PG = 6–phosphogluconate; PGA = 3–phosphoglycerate; Pi = inorganic phosphate; R5P = ribose–5–phosphate (adapted from Böhme, 1998).

The biological N_2 fixation is catalyzed by the O_2 irreversibly inactivated enzyme, Nase (Mohapatra and Gresshoffa, 1984), which is confined to a micro–anaerobic environment in heterocyts of the filamentous cyanobacteria *Anabaena*, under diazotrophic conditions. Gaseous N_2 reacts with H^+ generated from PSII with Nase as the catalyzer. In this reaction, ATP is essential and is transformed to ADP and Pi. In the light, ATP is formed by cyclic photophosphorylation mediated by PSI. Ferredoxin could be also photo-reduced by PSI at the expense of H_2 and NAD(P)H as electron donors (Böhme, 1998). The electron transport chain to Nase in heterocyts comes from the uptake hydrogenase, which couples poorly with the respiratory chain in heterocyts and does not function in CO_2 fixation by vegetative cells (Benemann and Weare, 1974a). As by-product, H_2 is produced at the same time. Actively growing N_2–fixing cultures of *A. cylindrica* can simultaneously evolve H_2 and O_2 from water and light. It has been suggested that N_2 fixation has potential use in solar energy conversion (Benemann and Weare, 1974b). That's why there are also many researches about H_2 production by cyanobacteria, which is warmly studied in new energy field.

Heterocyts, sites of N_2 fixation in certain filamentous cyanobacteria, are limited to a heterotrophic metabolism, rather than the photoautotrophic metabolism characteristic of

vegetative cells (Summers et al., 1995). It's most likely that the oxidative pentose phosphate pathway (PPP) represents the essential catabolic route of providing reductant for N_2 fixation and respiration in differentiated heterocyts and for dark growth of vegetative cells. In intact *A. cylindrica*, the energy requirement for N_2 fixation *in vivo* is also reported. Ferredoxin is indicated to be the reductant for N_2 fixation in the whole cells (Bothe and Loos, 1972), which in all probability, is likely to be reduced in a dark reaction and independently from the photosynthetic electron transport (Bothe and Loos, 1972). In the dark, reductant for N_2 and O_2 is generated by the activity of the oxidative polyenylphosphatidylcholine (PPC) and possibly by isocitrate dehydrogenase. NADPH thus formed donates electrons via ferredoxin NADP reductase (FNR) to a heterocyt-specific ferredoxin (FdxH) and then to the two components of Nase (Fe-protein and FeMo–protein) as indicated. NAD(P)H and H_2 are also electron donors to the respiratory electron transport generating the necessary ATP for the Nase reaction (Böhme, 1998).

In a cell–free system derived from heterocyts, the following substrates support Nase activity: glycogen, maltose, sucrose, glucose and fructose. Among these substances, some are particularly active, including glucose–6–phosphate (G6P), dihydroxyacetone phosphate (DAP), glyceraldehyde 3–phosphate (GAP) and fructose–1, 6–bisphosphate (FBP). Glycolytic substrates are inactive or inhibitory in acetylene reduction by heterocyt extract, such as phospho–enolpyruvate (PEP) and pyruvate (Böhme, 1998).

1.2.2 Structure, composition, related genes and inhibition of Nase

1.2.2.1 Structure, composition, related genes of Nase

As the catalyzer of biological N_2 fixation, Nase is well studied. The structure of Nase is shown in **Figure 1−7.** Each Fe protein molecule (shown at the top left and bottom right of the complex in brown) docks directly over the interface between an α/β subunit pair of the MoFe protein (in black and gray), which occupies the center of the structure, to juxtapose its [4Fe–4S] cluster (in yellow) with a P cluster (in red) at this interface. One FeMo cofactor (in pale blue) is accommodated within each α subunit. The two β subunits (in gray) provide the interactions among the two α/β subunit pairs (Schindelin et al., 1997) .

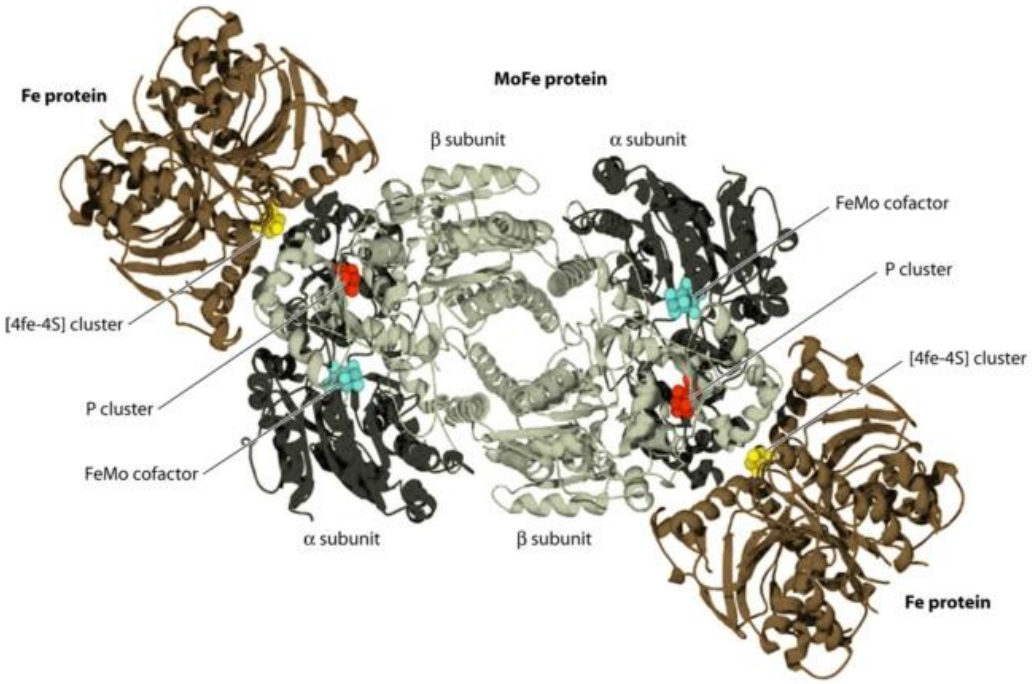


Figure 1−7. The structure of the 2:1 Fe protein-MoFe protein complex of the *Azotobacter vinelandii* Nase stabilized by MgADP plus $AlF4^-$; (adapted from Schindelin et al. 1997).

The concept of a common origin of N_2 fixation is consistent with the strikingly similar physical and chemical characteristics of N_2–fixing enzyme system present in otherwise dissimilar organisms (Fay, 1992). The enzyme complex consists of two component proteins. One is a Mo–Fe protein, called Nase, and the other is a Fe–containing protein, i.e. Nase reductase. Nase contains four (4Fe–4S) clusters and two molecules of Mo–Fe cofactor. The Mo–Fe cofactor is an essential component of Nase; it contains eight Fe and six S atoms per Mo atom.

Among the well-studied cyanobacteria, *A. variabilis* possesses three nitrogenases, two of them are expressed in heterocyts, a conventional Mo–Nase and an alternative V–Nase. Synthesis of these two enzymes is repressed in cells growing with a source of Nr; however, the V–Nase is also repressed by Mo. Expression of V-Nase is not affected by V, and expression of the Mo–Nase is not affected by the presence or absence of either Mo or V (Thiel et al., 1997; Thiel and Pratte, 2013). The structure of V–Nase is similar to that of Mo–Nase; however, it has a FeV cofactor in place of the FeMo cofactor found in Mo–Nase. Besides, a second Mo–dependent Nase is observed in vegetative cells, which expresses long before heterocyts formed under anaerobic conditions (Thiel et al., 1997).

The genes responsible for the express of the three Nases in *A. variabilis* are also completely studied, such as nifH1, nifD1, vnfH, vnfDGK and nifK1 (Thiel et al., 1997). The nifH1 gene, encoding the Nase reductase of the Mo–Nase which is expressed only in

heterocyts, is cotranscribed with nifD1 and nifK1, which together encode the Mo–Nase. These genes are expressed in the presence or absence of Mo or V. The vnfH gene, which encodes the Nase reductase of V–Nase, is located about 23 kb from vnfDGK, which encodes V–Nase; however, like vnfDGK, vnfH is expressed only in the absence of Mo, with or without V. Like nifH1, the vnfH gene is expressed exclusively in heterocyts under either aerobic or anaerobic growth conditions and thus is under the control of developmental factors. Besides, the gene cluster, nif2 encoding the second Mo–Nase exists in vegetative cells.

It is indicated that modification of Nase reductase develops as a result of decrease in carbohydrate-supported reductant supply of the heterocyts caused by carbon limitation or by increased diversion of carbohydrates towards NH_3 assimilation. Apparently, a product of nitrogen assimilation such as glutamine is not necessary for modification. The increase of O_2 concentration in heterocyts is a plausible consequence of all treatments causing Fe protein modification (Ernst et al., 1990b). Modification and demodification of Fe-protein are not coupled to the cell cycle since they follow darkening and illumination when the light or dark periods are changed (Ernst et al., 1990a). Reactivation of inactivated Nase occurs after restoration of diazotrophic growth conditions (Reich and Böger, 1989). In previously carbon limited cultures, reactivation has also been observed in the dark after addition of fructose and under anaerobiosis upon re-illumination in the presence of a photosynthesis inhibitor.

1.2.2.2 Inhibition of Nase by O_2

The enigmatic coexistence of O_2–sensitive Nase and O_2–evolving photosynthesis in diazotrophic cyanobacteria has fascinated researchers for decades. Research efforts have revealed a range of O_2 sensitivity of Nase in different strains of cyanobacteria and a variety of adaptations for the protection of Nase from damage by both atmospheric and photosynthetic sources of O_2. The most complex and apparently most efficient mechanisms for the protection of Nase are incorporated in heterocyts, the N_2–fixing cells of cyanobacteria (Fay, 1992). Genetic studies indicate that the controls of heterocyt development and Nase synthesis are closely interrelated and that the expression of N_2 fixation (nif) genes is regulated by O_2 partial pressure (pO_2).

In *A. variabilis*, the rate of acetylene reduction in the dark increased with increasing O_2 concentrations until a maximum value corresponding to 30 % O_2 in gas phase at 35°C, which presumably results from a requirement for energy provided by respiration. However, with illumination, *A. variabilis* grown at 0 % O_2 shows highest Nase activity. Both

photosynthesis and respiration contribute to Nase activity in *Anabaena* and their individual contributions depend on both O_2 concentration and temperature (Compaoré and Stal, 2010). Similarly, in *A. cylindrica*, inactivation of N_2 fixation at elevated temperature was a consequence of increased sensitivity to inhibition by O_2, which could be correlated with inhibition of uptake hydrogenase (Gallon et al., 1993).

1.2.2.3 Strategies for protecting Nase from O_2 inhibition

To offer a micro–oxic environment for Nase, various physiological, morphological and ecological strategies exist among different diazotrophs to overcome this dilemma in both directions of preventing O_2 from entering the cells and keeping the ability of consuming O_2 already in the cells (Bergman et al., 1986; Berman-Frank et al., 2005; Kangatharalingam et al., 1992).

On one hand, they turn out to be morphologically distinct cells, which can easily be distinguished from vegetative cells by their thick walls and by the generally homogeneous, usually pale yellowish content and are surrounded by a thick, firm envelope, consisting of an inner glycolipidic layer, followed by a laminated, polysaccharidic, homogenous central layer and outer fibrous layers. It has been suggested that four distinct glycolipids in the laminated layer of the heterocyt envelope provide a passive barrier to O_2 diffusion (Haury and Wolk, 1978; Lambein and Wolk, 1973). The structure of these lipids has been studied showing that Lipids III and IV consist of a hexose bound by a glycosidic linkage to the terminal hydroxyls of the long-chain polyhydroxy alcohols, while Lipid I consists of hexose bound to the carboxyl group of 25–hydroxyhexacosanoic acid and Lipid II appears to be structurally similar to lipid I. Lipid III as the major lipid fulfills the space between two hydrophobic hydrocarbon chains which may be closely packed with the hydrophilic ends as the top and bottom of each lamina. This assemblage might provide efficient barrier for heterocyts to the penetration of lipophobic chemicals and maybe O_2 (Lambein and Wolk, 1973). The mutants deficient in these envelope glycolipids are detected to have little or no Nase activity when assayed aerobically, while O_2–sensitive mutants with normal glycolipid composition are presumed to have genetic lesions in O_2 protection mechanisms (Haury and Wolk, 1978).

As it is reported, the increase of exogenous O_2 partial pressure (pO_2) from 5 to 40 kPa has induced thickness of the heterocyt envelope of *A. flos-aquae*, the majority of which occurred in the glycolipid layer area of the envelope (Kangatharalingam et al., 1992), (**Figure 1−8)**. Such thickening appears to be an O_2-induced mechanism for providing a greater O_2

diffusion barrier against O_2 inhibition of Nase. Otherwise, the decrease of Nase activity at 20 kPa pO_2 indicates that a thickened envelope is not enough for preventing O_2 diffusion (Kangatharalingam et al., 1992). Heterocyt frequency of *A. cylindrica* under highly elevated CO_2 levels with no O_2 has been shown to be higher than that in air (Kulasooriya et al., 1972), while that was the lowest at atmospheric O_2 pressure 20 kPa (Kangatharalingam et al., 1992).

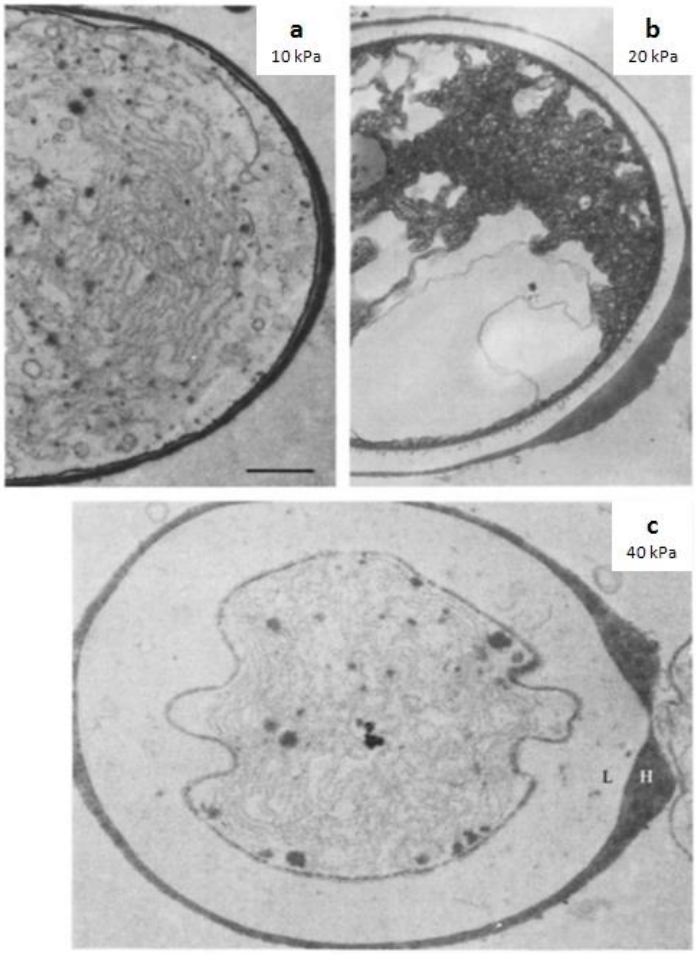


Figure 1−8. Transmission electron microscopy of *A. flos-aquae* grown at different levels of pO_2 ; **(a)** 10 kPa, **(b)** 20 kPa, **(c)** 40 kPa; (adapted from Kangatharalingam et al., 1992).

Another big change in the structure of heterocyts is that there are polar nodules at one or both ends of the heterocyt which adjacent to vegetative cells. Heterocyts and vegetative cells are connected by special pores named polar nodules, which are morphologically visible at both ends (in intercalary heterocyts) or only at one end (in heterocyts in polarized trichomes) in the thickened cell wall (Lang and Fisher, 1969). As it is known, the cell wall of heterocyts are not permeable for gases and liquids, the polar nodules (terminal pores) represent the main contact with environment. Researchers also propose that (Walsby, 2007) the main gas–diffusion pathway is through the terminal pores, transmembrane proteins of which would make the narrow pores permeable enough and might provide a means of regulating the rate of

gas exchange, increasing it by day, when N_2 fixation is most active, and decreasing it at night, minimizing O_2 entry. Walsby measuring the gas permeability of heterocyts and calculating the internal gas concentration, proposes that there are several ways in which the permeability of the pore might be modified: the periplasmic space might change in answer to increasing pressure or the periplasm lining the neck might be obstructed, the diffusion to the junction membrane might be blocked by the cyanophycin plug or the gas permeability might be altered by changing the concentration of proteins in membranes at the heterocyt junction (Walsby, 2007). By comparing the regulation of gas exchange through heterocyt pores and leaf stomata in certain C4 plants, he also presumes that they have similar working mechanism (Walsby, 2007).

A membrane-associated manganese superoxide dismutase (MnSOD) of the *Anabaena* sp. PCC 7120 is revealed to present in vegetative cells and heterocyts and that reactive O_2 species produced in heterocyts under aerobic conditions cause the inactivation of Nase in the absence of MnSOD (Zhao et al., 2007). Cyanoglobin, a heme protein encoded by glbN gene with high affinity for O_2, is found in many *Nostoc* strains, but some of them include a few symbiotic strains, lack the protein (Hill et al., 1996). GlbN, found solely in the soluble fraction of cell extracts, has a specific and prominent subcellular location around the periphery of the cytosolic face of cell membrane *in vivo*. When the rate of O_2 evolution is repressed, maximum accumulation of glbN takes place in both heterocyts and vegetative cells of N_2–fixing cultures, which coincided with maximum synthesis of NifH and ferredoxin $NADP^+$. So, glbN is presumed to be able to scavenge O_2 and to be a component of a membrane–associated microaerobically induced terminal cytochrome oxidase (Hill et al., 1996).

On the other hand, heterocyts also dismantle their O_2–making machinery for protecting Nase from O_2 inhibition. They lose a large portion of PBPs, their PSII center, and ribulose biphosphate carboxylase (Peters, 1989), ensuring that they don't generate O_2 or fix carbon (Ke and Haselkorn, 2013 ; Tel-Or and Stewart, 1977). The role of respiratory activity in protecting Nase from O_2 that diffuses into the heterocyt is studied by inhibiting carbon metabolism. The degradation of the PBS during Nr deprivation possibly initiates at the linker between APC and PSII (Ke and Haselkorn, 2013).

In addition, it has often been suggested, that the thickened envelope so extensively restricts the entry of O_2, that the O_2 which enters can be assimilated by oxidative reactions taking place inside heterocyts such that a very low concentration of O_2 is maintained in the interior (Gallon and A. Falah Hamadi, 1984). The ability of some cyanobacteria to grow

diazotrophically in the dark under aerobic conditions (Wolk et al., 1976) provides evidence for the existence of respiratory processes in heterocyts, which has the function of lowering the concentration of O_2 and providing energy in the form of ATP. Respiration is based on the organic substances imported from the adjacent vegetative cells. These substances can also alternatively provide reductants for N_2 fixation (Wolk et al., 1994). It is no doubt that respiration consumes O_2 then produce water, which could help to protect Nase from inactivation by O_2 (Murry et al., 1984 ; Walsby, 2007). Another role of respiration in heterocyts might be to produce ATP for N_2 fixation.

Until now, we have known that heterocyts developed several strategies for their special role fixing atmospheric N_2, by erecting thick and firm envelop around their cell wall, by generating polar nodules at the ends connecting with vegetative cells, by degrading O_2 producing metabolism PSII and by remaining O_2 consuming activity of respiration. Moreover, the reaction of N_2 fixation and structure of the catalyzer Nase are already introduced. Then, we will have an overview of the product of N_2 fixation, NH_3, about their output pathway.

1.2.3 Strategy for extracellular NH_3 production

1.2.3.1 Employment of MSX

Until now, several ways to produce extracellular NH3 have been reported (**Table 1−2**). The most frequently used but toxic way is to block the activity of GS enzyme by adding MSX (an inhibitor of GS), with which NH_3 cannot react with glutamate to form glutamine and as a result, NH_3 will be released out of the cells.

As the primary product of biological N_2 fixation, up to 90 % of NH_3 can be released extracellularly if the primary enzyme GS is inhibited by glutamate analogues such as MSX (Stewart and Singh, 1975). Among several methionine derivatives, MSX and methionine sulfone are the most effective inhibitors of GS. The present studies show that inhibition by methionine sulfone is reversible and that this inhibitor does not remain attached to the enzyme. In contrast, the inhibition of GS by MSX is irreversible and is associated with the binding to the enzyme (close to 8 moles MSX per mole GS). Since GS is composed of eight apparently identical subunits (Ronzio et al., 1969), these findings are in accord with the view that 1.0 mole of MSX binds to an active site of each subunit. Inhibition by MSX and its binding to GS require ATP and Mg^{2+} and are associated with the cleavage of ATP to ADP.

MSX is known to induce glycogen accumulation in astrocytes of the mouse cerebral cortex (Swanson et al., 1989). MSX at 1.0 mmol L^{-1} induced a 300 % increase in glycogen content. The time course of glucose and glycogen content after MSX administration suggests this increase to be the result of slowed glycogenolysis rather than accelerated glycogen synthesis (Swanson et al., 1989).

As it is reported, NH_3 release has been achieved by diazotrophic cultures of *Anabaena* strains in the presence of 1.0 μmol L^{-1} MSX under static culture conditions. NH_3 release from *Anabaena* 7120 and AnFPNhetR cells are respectively 2.5 and 5.5 μmol NH_3 mg Chl a^{-1} after monitored for 5 days (Chaurasia and Apte, 2011). Continuous NH_3 photoproduction lasting for one week has been carried out in a "trickling-medium" bioreactor using PV–immobilized *A. azollae* cells (Park et al., 1991). When cells are treated with 10 μmol L^{-1} MSX for pulse treatment during 20 min, NH_3 concentration in the eluates has reached 500 μmol L^{-1}, and no cell leakage was observed. Nase activity and NH_3 production are higher after MSX treatment, which has been showed to be able to prevent inactivation of Nase by exogenous NH_3 in *Anabaena* sp.

Table 1−2
Extracellular NH_3 productivity in the presence of MSX by various strains of *Anabaena.*

Species	Treatment	MSX (μmol L^{-1})	NH_4^+ productivity	Duration (h)	Reference
Anabaena PCC 7120	FL	55	94 μmol g $protein^{-1}$ h^{-1}	——	Singh and Tiwari, 1998
Anabaena PCC 7120-M	FL	55	70–77 μmol g $protein^{-1}$ h^{-1}	——	Singh and Tiwari, 1998
A. azollae	Asy	100	630 μmol g $protein^{-1}$ h^{-1}	> 4	Zimmerman and Boussiba, 1987
Anabaena 27893	Im	10	40 μmol mg Chl^{-1} h^{-1}	184	Kerby *et al.*, 1986
Anabaena 27893	Im	2	31 μmol mg Chl^{-1} h^{-1}	300	Kerby *et al.*, 1986
A. siamensis SS1	FL	500	8.7 μmol mg Chl^{-1} h^{-1}	120	Thomas *et al.*, 1991
A.variabilis PCC7937	FL	10	3.5 μmol mg Chl^{-1} h^{-1}	——	Bui *et al.*, 2014
*A.variab*ilis PCC7937	FL	25	1.9 μmol mg Chl^{-1} h^{-1}	——	Bui *et al.*, 2014
A.variabilis PCC7937-C9	FL	10	4.9 μmol mg Chl^{-1} h^{-1}	——	Bui *et al.*, 2014
A.variabilis PCC7937-C9	FL	25	4.5 μmol mg Chl^{-1} h^{-1}	——	Bui *et al.*, 2014

WT = Wild type; M = Mutant; Im = Immobilized; FL=Free living; As = Asymbiotic strain.

Even grown on NH_4Cl, *A. cylindrica* is still found to release NH_3 in the presence of MSX. In the light, the release is maximal at 0.2 mmol L^{-1} MSX. It is suggested that the major source of NH_3 released is the photorespiratory conversion of glycine to serine as increase in light intensity, glyoxylate and glutamate all stimulated the release while high CO_2 (3.0 %) lowered the release. Furthermore, a substantial part of the NH_3 released by N_2-fixing *A. cylindrica* in the presence of MSX may thus originate from the glycolate pathway (Bergman, 1984). In line with this report, GS is proved to be important in the assimilation of NH_3 in leaves and that the glycine-serine conversion is a major source of NH_3, since additions of

MSX result in an increase in NH_3 in seedling leaves of wheat and corn. The NH_3 assimilation rate of photorespiration is much higher in wheat than in corn leaves. Photorespiration as measured by the accumulation of NH_3 is 16 % of the apparent rate of photosynthesis (Martin et al., 1983). Exogenous NH_4Cl and MSX, which caused NH_4^+ accumulation in roots, inhibit root growth of rice seedlings. However, the growth inhibition of roots by MSX could be reversed by the addition of L–glutamic acid or L-glutamine (Lin and Kao, 1996).

By adding MSX, extracellular NH_3 release is initiated from an independent asymbiotic strain of *A. azollae*. NH_3–grown cells liberate NH_3 more slowly than remove from the medium. No optimum is observed between pH 5.0 and 9.0 for NH_3 movement into or out of cells of *A. azollae* (Zimmerman and Boussiba, 1987).

1.2.3.2 Immobilization

The environmentally friendly way for extracellular NH_3 production is the application of immobilization (Babu and S. Kannaiyan, 1998; Kannaiyan et al., 1997, 1994; Kerby et al., 1986; Wang et al., 1991), as shown in **Table 1−3**. Immobilization is the process of attaching cells or their constituent biocatalysts to a solid matrix so that they do not move independently when placed in a fluid environment. Immobilization could be carried out by physical means such as adsorption or entrapment in a gel or foam matrix or by chemical methods such as covalent bonding (Babu and S. Kannaiyan, 1998).

It is difficult to control the flow of nitrogen compounds needed for the development of rice plants. The breakthrough of this problem will be the development of cyanobacterial strains, which release ammonium continuously into the field. Ammonia excreting mutants have been isolated for cyanobacteria like *A. variabilis*, *Nostoc muscorum* (Singh et al., 1983; Spiller et al., 1986; Thomas et al., 1991). The process can be successfully enhanced by immobilizing these diazotrophic cyanobacteria on suitable matrices (Kannaiyan et al., 1994). It has been reported that immobilization of N_2–fixing cyanobacteria on solid substrates stimulated growth and ammonia excretion rather than in free living condition (Kannaiyan et al., 1994).

Table 1−3
Ammonium production under different immobilization conditions.

Strain	*A. variabilis*	*N. muscorum*	*A. azollae*	*A. variabilis*	*A. variabilis*
Medium	BG11o	BG11o	BG11o	BG11o	BG11o
T (°C)	28±1	28±1	——	30	25±1
Irradiance	3000 lux	3000 lux	65 μmol m^{-2} s^{-1}	220 μmol m^{-2} s^{-1}	45 μmol m^{-2} s^{-1} (18h/24h)
Duration (d)	14	14	15	7.5	7
Material	FL, PUF, SCW,PW	FL, PUF, SCW,PW	WPF	C-AGB	C-AGB
Size	0.5-cm pieces at 1.5 g	at 2.0 g	0.5 cm^{-3} cube at 2.0 g	5-50 μg Chl *a* g^{-1} beads	——
Autoclave	15 lb pressure at 120 °C, 30min	15 lb pressure 120 °C, 30min	125 kPa, 30 min	——	——
NH_3 production	70−100 μmol L^{-1}	80−140 μmol L^{-1}	>2500 μmol L^{-1}	56 μmol mg Chl^{-1} h^{-1}	53 μmol mg Chl^{-1} h^{-1}
Reference	Babu and S. Kannaiyan, 1998	Babu and S. Kannaiyan, 1998	Kannaiyan *et al.*, 1994	Kerby *et al.*, 1986	Singh *et al.*, 2013

FL = Free living; Av (MHR) Edar = Multiple herbicide ammonia excretory resistant strain of *A. variabilis*; PUF = Polyurethane foam; SCW = Sugarcane waste; PW = Paper waste; WPF = White polyvinyl foam; C–AGB = Ca–alginate gel beads.

Immobilized cyanobacterial cultures in solid matrices have showed relatively better growth when compared to those under free–living conditions, thanks to the use of perfusion photobioreactors. Immobilized cyanobacterial cells have also shown higher Nase activity comparing to free cells. Total Nase activities of cyanobacterial strains were reported to be 24 % − 66 % greater in different solid matrices than free living counterparts. Such efficiency was

related to the high biomass concentrations. However we cannot exclude an effect of the decrease of dissolved oxygen concentrations as volumetric photosynthetic activities are known to be reduced as a consequence of a decrease of light transfer induced by self-shading among high cell densities populations. Among the substrates, sodium alginate proved to be better immobilizing substrate (Singh et al., 2013). Samal and Kannaiyan (Samal and Kannaiyan, 1992) have shown good growth and heterocyt frequency in four isolates of *A. azollae* in the immobilized state in alginate. *A. azollae* and *A. variabilis*, when immobilized in polyurethane and polyvinyl foams or calcium alginate beads, have been shown to release extracellular NH_3 (Kannaiyan et al., 1997). Significantly higher quantities of NH_3 have been shown to be excreted by *Anabaena* sp. immobilized in polyurethane foam in the presence of MSX compared to controls.

Solid matrices such as polyurethane foam (PUF), sugarcane waste (SW), and paper waste (PW) have been used for immobilizing N_2-fixing cyanobacteria *A. variabilis* and *Nostoc muscorum*. Higher NH_3 excretion is obtained by immobilized cells in sugarcane waste than in PUF and paper waste and free-living cyanobacterial cultures. Maximum of NH_3 excretion by both strains has been achieved on the 14th day of inoculation, with lowest on the 28th day. The solid matrices also provide favorable conditions for colonization of cyanobacteria, which interestingly plays a vital role in increasing N_2–fixing activity and NH_3 production (Babu and S. Kannaiyan, 1998). *A. azollae*, isolated from *Azolla filiculoides* and grown in Nr–free medium, immobilized in polyvinyl foam pieces and incorporated into a PBR system produced NH_3 continuously in significant amounts (Kannaiyan et al., 1994).

Cell immobilization led to an increase and/or to a stabilization of the rate of NH_3 photoproduction. This increase occurred mainly via hydrogenase-mediated production (Brouers and Hall, 1986). Immobilization also can stabilize Nase activity and increase initial rates of N_2 fixation.

1.2.3.3 Mutagenesis

An alternative to the use of analogues which inhibit GS activity is to select for mutant strains partially deficient in GS activity, which can liberate NH_3 produced from N_2–fixation into the medium (**Table 1−4**). Mutagenesis, moderate to environment, has been tried to get mutants with the ability of excreting NH_3. An alternative method for obtaining GS deficient mutants is selection for resistance to the NH_4^+ analogues methyl ammonium and ethylene-diamine (EDA). Both of these compounds are toxic to cyanobacteria and can be metabolized

by GS to glutamine analogues which accumulate intracellularly (Kerby et al., 1986). It has been reported that certain mutant strains of cyanobacteria, such as *A. variabilis*, are capable of secreting NH_3 into the natural environment (Spiller et al., 1986).

EDA is toxic to *A. variabilis* and inhibits Nase activity. The inhibition of Nase can be prevented by pretreatment of cells with MSX. Mutant strains of *A. variabilis* (ED81, ED92), resistant to EDA, have low levels of GS biosynthetic activity compared to the WT strain, and they are routinely maintained in medium supplemented with 15 mg L^{-1} EDA (Kerby et al., 1986). ED92 had a low level of GS protein whereas ED81 had a similar level to that of the parent strain as estimated using antibodies against GS. Both strains are able to fix N_2 and liberate NH_3 into the medium. Following immobilization of the mutant strains, sustained photoproduction of NH_3 has been obtained in air–lift reactors at a rate of 50 μmol NH_3 mg Chl^{-1} h^{-1}, which is comparable to the rates obtained by immobilized cyanobacteria treated with MSX.

A stable mutant (SS1) of *A. siamensis*, isolated by ethyl methanesulfonate (EMS) mutagenesis, showed ability of releasing NH_3 to the medium (Thomas et al., 1991). In batch cultures, the NH_3 production rate peaks at the early log phase and gradually decreases until the 4^{th} day of growth when the cultures reached a density of 90 mg Chl L^{-1}. With continuous culture at a cell density of 5 mg Chl L^{-1}, constant release of NH_3 by SS1 at a rate of 40 μmol L^{-1} h^{-1} has been obtained up to 120 h. Alginate beads immobilization shows no impact on the activity of GS and Nase, but NH_3 excretion at the rate of 58 μmol L^{-1} h^{-1} has been obtained 20 h after loading the immobilized cells in bioreactors (Thomas et al., 1991).

Mutants of *Anabaena* PCC 7120 defective in GS activity isolated following transposon mutagenesis. Mutants M11, M55 and M73 show about 60% less GS activity in Nr–grown aerobic cultures than the wild–type strain and are resistant to MSX. They are proven to have the capacity to excrete NH_3 continuously into the culture medium and perform an enhanced level of aerobic Nase activity (Singh and Tiwari, 1998). GS deficient strains are considred to be usefu in liberating extracellular NH_3 without treatment of MSX (Kerby et al., 1986; Reglinski et al., 1989).

Table 1−4
Mutant strains of *Anabaena* and their NH_3 excreting abilities.

Strain	Mutant	Mutagenesis method	NH_3-excreting ability	Reference
A. variabilis (ATCC 29413)	nitrogenase derepressed mutant, SA-1	EMS mutagenesis	11.25 μmol L^{-1} h^{-1}	Spiller *et al.*, 1986
Anabaena sp. *PCC 7120*	Partially GS-defective mutants, M11, M55 and M73	Transposon mutagenesis	70-76 μmol g $protein^{-1}$ h^{-1}	Singh and Tiwari, 1998
A. variabilis	GS-defective mutants ED81 and ED92	15 mg L^{-1} EDA in medium	40-50 μmol mg Chl^{-1} h^{-1}	Kerby *et al.*, 1986
A. siamensis	A stable mutant named *A. siamensis* SS1	EMS mutagenesis	8.0 μmol mg Chl^{-1} h^{-1}	Thomas *et al.*, 1990
A. variabilis	*A. variabilis* PCC 7937-C9	EMS double–random mutagenesis	4.9 μmol mg Chl^{-1} h^{-1}	Bui *et al.*, 2014

EMS = Ethyl methanesulfonate; EDA = Ethylene–diamine; GS = Glutamine synthetase.

1.2.4 Problems of NH_3 excretion by N_2–fixing cyanobacteria

Conditions have been developed to lengthen the time during which photosynthetic N_2 fixation proceeds freely in batch cultures of *Anabaena* sp. ATCC 33047, whereas the subsequent conversion of NH_3 into organic nitrogen remains blocked, with the resulting NH_3 released to culture medium (Ramos and Losada, 1984). The biochemically manipulated cyanobacterial filaments represent a system that is relatively stable over time for the conversion of light energy into chemical energy, with the generation of a valuable fuel and fertilizer through the photoreduction of N_2 to NH_3. Maximal rates of NH_3 production (25 − 30 μmol mg^{-1} of Chl h^{-1}) are maintained for about 50 h when MSX is added every 20 h. After

that, NH_3 production ceases due to a deficiency of glutamine and other nitrogenous compounds in the filaments, followed by cell lysis. By adding a small amount of glutamine to or by allowing the cells to recover for 8 h in the absence of MSX after every 40 h period in the presence of the inhibitor, the effective NH_3 production period could be further extended to 7 days. A more prolonged steady production of NH_3 (longer than 2 weeks) can be achieved by alternating treatments with MSX and phosphinothricin, 8 h recovery periods in the absence of either compound should also be alternated throughout.

The use of glutamate analogues, such as MSX, to induce NH_3 production by cyanobacteria, although technically feasible has three main disadvantages. First, it is difficult to sustain prolonged NH_3 production in their presence in bioreactors; second, since the analogue is toxic it has to be separated from the extracellularly produced NH_3 prior to the use of the latter as a bio–fertilizer; third, MSX is a relatively expensive chemical, which costs around 1000 euro per gram as is given by the chemical selling company Sigma-Aldrich (http://www.sigmaaldrich.com/catalog/product/sial/76078?lang=fr&region=FR). Moreover, negative impacts of MSX on the growth of bacteria, plants and humans have been widely reported (Singh et al., 2008).

Moreover, it is difficult for immobilized cyanobacteria to maintain their producing ability of NH_3 excretion. Even though the mutant strains obtained by different mutagenesis methods, they would lose their NH_3 releasing capability after a period. In addition, the NH_3 productivity by cyanobacteria is still too low to be applied as bio–fertilizer. Thus, how to make cells produce NH_3 continuously with high productivity is the main problem to be solved. However, there is still long way to go to solve this problem. So, in the following part, the effect of some environmental parameters on the growth and NH_3 production will be analyzed in respective to literatures.

1.3. Effect of environmental factors on the growth of cyanobacteria and NH_3 production

To better understand the characteristics of N_2–fixing cyanobacteria and their ability in NH_3 production, the effect of growth conditions must was be analyzed firstly. Growth conditions will be discussed in several aspects, such as growing medium (the composition and pH), light intensity, temperature, gas (type and flow rate) and PBRs. Some parameters also affect the excretion of NH_3, such as pH, light intensity, micronutrient elements, MSX concentration etc.

1.3.1 Nutrients

1.3.1.1 Composition of culture medium

Until now, several media have been used for culturing N_2–fixing cyanobacteria. Here, AA medium, BW3 medium and the most common BG11 medium are presented in **Table 1−5** (Allen and Arnon, 1955 ; Bui et al., 2014 ; Silva et al., 1989 ; Stanier et al., 1971).

Cyanobacteria occupy most ecological niches on earth, being tolerant to a large number of environmental stresses, including salinity and drought. Many of them also fix atmospheric N_2. They are responsible for a significant share of bio–solar energy conversions on this planet and make substantial contributions to the carbon and nitrogen status of both oceans and soils.

Table 1−5
Examples of media ever used for the cultivation of N_2-fixing cyanobacteria.

Medium	AA	BW3	BG11	$BG11_o$*
Macro elements (mmol L^{-1})				
Na^+	4.0	154	18.0	0.018
K^+	2.0	12.9	0.46	0.23
PO_4^{3-}	1.0	1.45	0.23	0.12
Mg^{2+}	1.0	1.5	0.3	0.3
SO_4^{2-}	1.0	1.5	0.3	0.3
Cl^-	1.0	0.54	0.48	0.48
Ca^{2+}	0.5	0.27	0.24	0.24
NO_3^-	0.16 (μM)	10	17.6	0
CO_3^{2-}	0	54	0.19	0
Micro elements (μmol L^{-1})				
EDTA	10.90	163	1.91	3.27
Fe^{3+}	10.90	88.8	21.43	3.27
NH_4^+	0.09	0	21.43	0
B	8.09	57.15	46.26	46.26
Mn	2.96	17.94	10.71	9.15
Zn	0.17	15.65	0.77	0.77
Cu	0.08	8	0.32	0.32
Mo	0.69	10.33	1.61	1.61
V	0.09	0.98	0	0
Co	0.03	5.25	0.17	0.21
Ni	0.04	0.71	——	——
Cr	<0.01	——	——	——
W	0.03	——	——	——
Ti	<0.01	——	——	——
pH	nature	7.7	7.1	nature
Reference	Allen and Arnon, 1955	Silva *et al*., 1989	Stanier *et al*., 1971	Bui *et al*., 2014

* refers to the medium used in the thesis.

From **Table 1−5**, it's easy to see that the nutrient composition has been simplified. As important compositions, Na, K, Ca, Mg, P are the macro–elements, while the micro–elements contain Fe, Mn, Zn, Cu, B, Mo, Co and others. Fogg concluded that the excretion of nitrogenous substances is also favored by deficiencies of micronutrient elements, especially iron ((Fogg, 1949). Temperature and pH are separately around 30 °C and 7.0, while researchers use different light intensities for various studies.

1.3.1.2 Sodium

Sodium (Na^+) and potassium (K^+) are two of the most prevalent cations on the earth. While K^+ is an essential macronutrient in most life–forms, Na^+ is strongly discriminated by means of highly selective alkali cation transport systems, favoring K^+ over Na^+. *A. variabilis* and *Anacystis nidulans* have clear-cut requirements for both Na^+ and K^+ (Kretz and Myers, 1955). Without added Na^+, logarithmic growth is not maintained; addition of NaCl to the deficient cultures elicits complete return to logarithmic growth. Similar results have been obtained for *Nostoc muscorum* with Na^+ and for all three algae with K^+. Addition of 40 ppm Na^+ will support maximum growth while 4 ppm will not; addition of 5 ppm K^+ will support maximum growth while 0.5 ppm will not. The Na^+/K^+ ratio varied from 40/800 to 800/5 has no effect on their growth rate (Kretz and Myers, 1955).

Evidence from chloramphenicol treatment of the cells suggests that Na^+ may exert its control of nitrate reductase through a protein factor (Brownell and Nicholas, 1967). *A. cylindrica* grown in the presence of nitrate requires higher levels of Na^+ (0.4 mg L^{-1} NaCl) to prevent chlorosis than when grown without Nr. The loss of chlorophyll has been proven to be caused by nitrite toxicity, since Na^+ deficiency led to clear increase in the nitrate reductase and amounts of nitrite accumulated when lack of Na^+ resulted in chlorosis. Various nitrogenous compounds including NH_4Cl, amides and amino acids at low concentrations (0.1 mmol L^{-1}) have greatly reduced the nitrite accumulation in Na^+–deficient cultures within 20 hours (Brownell and Nicholas, 1967). They also find that *A. torulosa* requires Na^+ for growth on N_2 but not in the presence of NH_4^+, which is in contrast with the chlorosis resistance ability of *A. cylindrical* (Brownell and Nicholas, 1967). Nonetheless, higher doses of salinity (0.5 mol L^{-1}) can also cause reduction in growth criteria of *A. circinalis,* such as absorbance, chlorophyll content and protein content, with the time elapses, and caused increasing in carbohydrate content, significant increasing in proline content has also been recorded (Hifney, 2013).

It has also been indicated that N_2 fixation in Na^+ deficient cells is reduced. Since the incorporation of NH_3 or glutamate into protein has been increased under this condition, it is likely that Na^+ is required for the conversion from N_2 to NH_3 (Brownell and Nicholas, 1967). In consistence, it was reported that Na^+-starved cultures of *A. torulosa* differentiated normal heterocyts, but showed relatively low or no Nase activity, which appears rapidly with the addition of Na^+. The time course of appearance of activity after addition of Na^+ suggests that

Na^+ is involved in activation of the existing enzyme rather than in its de novo synthesis (Apte and Thomas, 1980).

Apte and Thomas have furtherly proven their former observations that Na^+ is required for Nase activity and diazotrophic growth by *A. torulosa* and that Na^+ addition to cultures deficient in Na^+ can restore Nase activity, but heterocyt differentiation and Nase synthesis are unaffected by Na^+. The same phenomenon has been observed in non-heterocytous cyanobacterium *Plectonema boryanum*, indicating that in cyanobacteria Nase is synthesized even in the absence of Na^+ but functions only in its presence (Apte et al., 1987). They also find that the curtailment of Na^+ influx, effected by certain environmental factors (such as alkaline pH, K^+ at concentrations above 25 mmol L^{-1}, NO_3^-, and NH_4^+), is a major mechanism of salt tolerance in cyanobacteria (Apte et al., 1987).

Similar result has also been documented in cyanobacteria concerning an unusual, specific and absolute requirement for trace quantities of Na^+. It has long been believed that cyanobacteria scavenge and immobilize Na^+, but Apte and Alahari have shown that Na^+ exclusion brought about by curtailment of influx and active efflux of Na^+ forms the basis of salt tolerance and that the inherent salt tolerance can be modified by factors that modulate Na^+ fluxes in cyanobacteria (Apte and Alahari, 1994).

Effect of Na^+ on HCO_3^- transport in *A. variabilis* (Reinhold et al., 1984) indicates that HCO_3^- transport is specially strongly promoted by Na^+. With kinetic analysis, they have raised the possibility that the transport is a $Na^+ – HCO_3^-$ symport, or a $Na^+ – H^+$ antiport (or $Na^+ – OH^-$ symport) system mediating the efflux of OH^- ions derived from the entering HCO_3^- ions. Similar study has also been done with unicellular cyanobacterium *Synechococcus leopoliensis* (Espie and Canvin, 1987), showing that growth conditions influence the mode by which *Synechococcus* acquired HCO_3^- for subsequent photosynthetic fixation. With analysis of ^{14}C-fixation during isotopic disequilibrium, it has been proven that standing culture cells possess both Na^+ – independent and Na^+ – dependent CO_2 transport systems, the conversion from the former to the latter could be accomplished by transferring cells grown in standing to growth in cultures bubbled with air. That is to say, the photosynthesis and HCO_3^- uptake are Na^+ – dependent by air–grown cells, but not Na^+ – dependent by standing culture cells (Espie and Canvin, 1987).

Besides, Na^+ also affects nitrate uptake. In *Synechocystis* sp. strain PCC 6803, Na^+ can induce an increase in nitrate uptake, while NH_4^+, Cl^-, and dl-glyceraldehyde will cause a reduction (Baebprasert et al., 2011). Na^+ also stimulates phosphate uptake both in Pi-sufficient cells and Pi-starved cells which follows Michaelis-Menten kinetics, and half–

saturation (K½) of phosphate uptake has been reached with a Na^+ concentration of 212 μmol L^{-1}. This indicates the existence of a Na^+–dependent phosphate uptake in *Anabaena* PCC 7119 (Valiente and Avendafio, 1993). The absence of Na^+ reduces the rates of phosphate uptake at all phosphate concentrations assayed (1 − 20 μmol L^{-1}), but does not change significantly the concentration of phosphate required for reaching half-saturation (K½).

What's more, light–induced acidification by *A. variabilis* has been shown to be Na^+–dependent with an optimum concentration of 40 − 60 mmol L^{-1} (Scherer et al., 1988b). The nature of the light-induced, Na^+–dependent acidification of culture medium and Na^+ uptake by *Synechococcus* has been afterward studied, suggesting that the acidification may stem from the activity of a light dependent proton excreting adenosine triphosphatase (ATP), while Na^+ transport seems to be mediated by both Na^+/H^+ antiport and Na^+ uniport (Kaplan et al., 1989). Na^+ uptake and acidification rate are strongly affected by medium pH, with the optimal pH for both processes being in the alkaline pH range; whereas the rate of acidification (net H^+ efflux) is strongly and specifically stimulated by Na^+. The uptake of Na^+ is stimulated by light and inorganic carbon, but the stimulation by inorganic carbon is observed only when it is present at the time when Na^+ is provided (Kaplan et al., 1989).

1.3.1.3 Potassium

As another essential nutrient, K^+ has important influence on the growth and differentiation of cyanobacteria. For example, addition of K^+ to the deficient cultures elicits complete return to logarithmic growth, whereas no logarithmic growth is maintained with the culture of A. variabilis, *Anacystis nidulan* and *Nostoc muscorum* G without added K^+ (Kretz and Myers, 1955). 2.0 mmol L^{-1} KCl addition to the culture medium can induce higher heterocyt frequencies at the end of the exponential phase (Fogg, 1949). When *A. flos-aquae* is grown in chemostat with phosphate-limiting growth and dilution rate of 0.36 − 0.72 d^{-1}, the presence of 15 mmol L^{-1} KNO_3 will increase the yields of cells to 2 folds and the alkaline phosphatase activity 20 folds (Bone, 1971). The deprivation of K^+ triggers akinete development in the cyanobacterium *Aphanizomenon ovalisporum* and *A. crassa*, but the highest akinete concentration is achieved at conditions that supported vegetative growth. Akinete formation is initiated 3 − 7 days after an induction of K^+ depletion, followed by 2 − 3 weeks of a maturation process (Sukenik et al., 2013). Similarly, by omitting K^+ from the growth medium, multiple metabolic impairments and cessation of growth of the filamentous, heterocytous, N_2–fixing cyanobacterium *A. torulosa* during both diazotrophic and nitrogen-

supplemented growth have been observed, which includes loss of photosynthetic pigments, impairment of photosynthetic functions, reduced synthesis of Nase reductase (Fe-protein), and inhibition of Nase activity (Alahari and Apte, 1998). All the observed metabolic defects are reversible.

Support has been added to that, in *A. variabilis* (ATCC 29413), K^+ transport is energy-dependent and may be mediated by different mechanisms at low and high external K^+ concentrations (Reed et al., 1981a). K^+ translocation was also found to be affected by external pH, saying that trans-plasmalemma K^+ fluxes and internal K^+ concentration were increased at high pH (Reed et al., 1981b). Rapid accumulation of K^+ during salt/osmotic stress has been observed in several cyanobacteria. Early K^+ responses to salt/osmotic stress have been identified to be membrane-bound in *Anabaena* strains (Apte and Alahari, 1994). K^+–channel genes are widespread and found in nearly all the free-living bacteria. It is speculated that prokaryotic K^+ channels function to allow adaptation to environmental and metabolic changes, although the actual roles of these channels in prokaryotes are not yet known (Kuo et al., 2005).

1.3.1.4 Calcium

It has been reported that calcium (Ca^{2+}) is essential for N_2 fixation by *Azola vinelandii* and *Nostoc muscorum*, but not for their growth in the presence of an adequate supply of Nr, and trace amount of Ca^{2+} present in the medium is adequate for the fixation (Allison et al., 1937; Burk and Lineweaver, 1931). In consistence, twenty years later, Allen and Arnon found that Ca^{2+} affects the growth of *Anabaena* to a larger scale in the absence of Nr than in its presence. Without addition of Ca^{2+}, *Anabaena* almost cannot grow in the absence of Nr even during 8 days, but it can grow to about 1.8 g L^{-1} dry weight in only 7 days in the Ca^{2+} – enriched medium. However, with an addition of more than 5 ppm Ca^{2+}, cells can grow better in absence of Nr (Allen and Arnon, 1955).

Ca^{2+} also mediates the regulatory process which modulates heterocyt frequency and the maintenance of cell structure in *Anabaena*, since 1.0 mmol L^{-1} Ca^{2+} is found to be the optimum for both the growth and the development of heterocyts, while 10 mmol L^{-1} and 100 mmol L^{-1} are proven to be, respectively, inhibitory and lethal for the growth (Singh and Mishra, 2014). At higher concentration of 100 mmol L^{-1} Ca^{2+}, heterocyt frequency is decreased and abnormal cell structures are formed. Since higher doses of salinity could cause reduction in growth criteria and reduction in protein content of *Anabaena*, the addition of Ca^{2+}

to salinized culture has efficiently improved the growth of cells and the K^+/Na^+ ratio, as well as protected the organism from the toxic effect of NaCl (Hifney, 2013).

1.3.1.5 Phosphorus

Phosphorus (P) is an essential element for the growth of all organisms, and is one of the proximate limiting elements for the growth of cyanobacteria in many natural habitats on land, in the ocean and in fresh water, and is generally the ultimate limiting element for primary productivity. Although RNA can account for over half of the non–storage phosphate in photosynthetic organisms, some primary producers have more ribosomes than the minimum needed for the observed rate of net protein synthesis; some of them may be needed for protein turnover. Two cases of protein turnover, which can occur at a much faster rate than the bulk protein turnover, are those of photo damaged PSII and O_2-damaged Nase. For photosynthetic diazotrophic organisms growing under phosphate limitation, there is a general trend in published data towards lower phosphate use efficiency with N_2 as their nitrogen source, rather than with Nr (Raven, 2012). The growth rate of *A. variabilis* has been shown to be closely related to phosphate concentration that linear relationship is found in the range of 21–55 mg L^{-1} of initial phosphate (Yoon et al., 2008). What's more, phosphate-starved cells are more sensitive to the toxic effects of arseniate than unstarved cells, but phosphate could alleviate some of the toxicity (Thiel, 1988). The same observation has also been obtained with *Anabaena* PCC 7119 that phosphate-starved cells take up phosphate at higher rates (Valiente and Avendafio, 1993). Additionally, phosphate is essential for akinete maturation and P–limitation restricts the number of mature akinetes (Sukenik et al., 2013).

In the recently, insoluble phosphate resources, Mussorie Rock Phosphate (MRP) and Tricalcium Phosphate (TCP), have been tested for cyanobacteria cultivation within *A. variabilis* (Yandigeri et al., 2010). *A. variabilis* is observed to proliferate better in the presence of TCP. In general, Acetylene Reduction Activity (ARA) in the presence of TCP is always higher than that in presence of MRP at all concentrations (Yandigeri et al., 2011). However, *A. variabilis* showed higher soluble protein content at 20 mg phosphorus (MRP), while maximum ARA is recorded at 20 mg phosphorus (TCP) followed by activity in the presence of soluble phosphorus (K_2HPO_4). The greatest N_2 fixation is obtained with 0.05 % K_2HPO_4, while a concentration of 0.5 % is somewhat toxic. In other experiments, there are some indications that even a concentration of 0.1 % may be above the optimum (Allison et al., 1937).

In *Anabaena* PCC 7119, phosphate uptake is found to be energy–dependent and Na^+–dependent both in phosphate-sufficient cells and P–starved cells, which follows Michaelis–Menten kinetics. phosphate uptake rates is reduced but not affected by pH in the absence of Na^+, whereas is affected by the pH in the presence of Na^+ with the optimal rates at pH 8.0 (Valiente and Avendafio, 1993). With P and nitrate in the growth–limiting range, no change in *Anabaena* growth rates is observed at N/P ratios from 1000 to 10. *Anabaena* has an efficient phosphate–uptake system, but its main strategy for growth in low–phosphate environments is presumed to depend on the storage of phosphorus during periods of abundant phosphorus supply (Nalewajko and Murphy, 2001). Concerning the transport of phosphate, a new transport system has been suggested to be induced by phosphorus starvation because cells of *A. variabilis* starved for phosphate for 3 days took up phosphate at about 100 times the rate of unstarved cells, which could be quickly repressed by addition of Pi (Thiel, 1988).

Additional work is needed to examine the generality of a statistically verified decrease in P use efficiency for diazotrophic growth related to their growth on other nitrogen sources and, if this is confirmed, further investigation of the mechanism is needed. The outcome of such work would be important for relating the global distribution of diazotrophy to phosphorus availability. No potassium acquisition mechanisms are known specific to diazotrophs up to now.

1.3.1.6 Other elements

For diazotrophic cyanobacteria, magnesium (Mg^{2+}) is surely essential for both their growth and N_2 fixation, as would be expected since this element is a constituent of chlorophyll (Allison et al., 1937). Mg^{2+} has responsible effect on the glucosyltransferase activity based on the study of the synthesis of glyceroglycolipids in membrane and soluble fractions of *A. variabilis* (Sato and Murata, 1982).

Molybdenum (Mo) is an element with five oxidation states; however, only the soluble Mo (VI) form can be transported and metabolized by cells. Bortels (1940) has shown that Mo is necessary for the growth of *A. cylindrica* in the absence of available Nr. The availability of Mo controls the expression of both Mo–Nase and V–Nase, while vanadate (V) had no effect on the expression of either Nase (Thiel and Pratte, 2013). It is suggested that the Mo-Nase is primarily responsible for the residual growth of *A. variabilis* since N_2 fixation can continue for many generations in Mo-starved and V–starved cells of both the wild–type strain and a

mutant lacking of V–Nase, but no growth has been observed in the mutant lacking Mo–Nase in absence of these two metals.

The effect of Mo concentration upon the growth of *A. cylindrica* has also been studied. Within the range of Mo concentration, it is found that the element does not affect the growth on NH_4Cl, but it is required for healthy growth on nitrate or N_2, respectively at an optimal concentration of 0.075 ppm and 0.20 ppm (Wolfe, 1954). For *A. torulosa*, Mo is also required for Nase activity and diazotrophic growth, since by adding Mo to Mo–deficient cultures Mo–Nase activity could be restored. However, Mo deficiency is able to enhance heterocyt differentiation (Apte and Thomas, 1984). Similarly, the Mo–Nase genes are evidenced to be made in the absence of Mo, and slow but persistent growth of cells in a medium with less than 10^{-10} mol L^{-1} Mo has been observed, but the enzyme cannot function in the absence of the Mo-cofactor (Thiel and Pratte, 2013). Both Mo–Nase and V–Nase function in heterocyts, but not usually at the same time, because V–Nase is not made in the presence of Mo (Pratte et al., 2006). V–Nase is capable of further reducing ethylene to ethane, which is not characteristic of Mo–Nase (Dilworth et al., 1988). By studying the effect of Mo concentration on transcription and translation of a putative Mo–storage protein (Mop) in *Nostoc* sp. PCC 7120, it is suggested that Mop does not store excess intracellular Mo, but may serve an unknown physiological function in Mo–limited metabolism (Glass et al., 2013).

Boron (B) has no appreciable beneficial effect on the growth of cyanobacteria (Allison et al., 1937), but the lack of boron inhibits Nase activity when cells of *Anabaena* sp. PCC 7119 grow depending on N_2 fixation (Garcia-gonzalez et al., 1988). However, the effect is not observed under anaerobic conditions or in the presence of Na–dithionite. Nase synthesis and heterocyt number are not affected by boron deficiency, but some dramatic changes in heterocyt morphology will appear that the activity of those enzymes related to the maintenance of low intracellular level of toxic oxygen species increased, including superoxide dismutase, catalase and peroxidase, which support the hypothesis of the role of boron in heterocyt envelope stabilization (Garcia-gonzalez et al., 1988).

The impact of titanium dioxide ($nTiO_2$) nanomaterials exposure on cell growth has been comprehensively investigated in *A. variabilis* at various dose concentrations and exposure time lengths, showing that both growth rate and N_2 fixation activity can be inhibited by $nTiO_2$ exposure, and that the time of exposure has a greater influence than $nTiO_2$ concentration in toxicity (Cherchi and Gu, 2010).

1.3.1.7 Organic substances

Sunlight is a satisfactory source of energy for providing the materials needed for respiration and cell growth of cyanobacteria, but the growth rate is usually accelerated by the addition of suitable sugars. In *Nostoc*, both sucrose and glucose have increased the growth in light (Allison et al., 1937). In similar, glucose is evidently assimilated since growth in older cultures of alga is increased in its presence (Fogg, 1949). However, glucose frequently produces toxic effect, owing to its decomposition during the autoclave when sterilized in an alkaline medium. So, separate sterilization of the glucose in a neutral or preferably slightly acid solution is advisable (Allison et al., 1937). Fogg (1949) has also reported that the supply of assimilable sources of organic carbon tends to increase heterocyt formation in *A. cylindrica* Lemm (Fogg, 1949).

Especially adding fructose to the growth medium led to a dramatic enhancement of *in vitro* cytochrome c oxidation and *in vivo* respiratory activity in *A. variabilis* ATCC 29413 without significantly influencing gene expression (Pils et al., 2004). Lang *et al.* (1987) have found that vegetative cells and young heterocyts of *A. variabilis* are significantly larger and more broadly oblong, filled with glycogen granules, and have fewer thylakoids, when 40 mmol L^{-1} fructose is added to the culture medium. Besides, developing heterocyts contain large numbers of glycogen granules with envelope formed precociously (Lang et al., 1987). Addition of fructose is found to be able to increase Nase activity even in darkness and caused demodification of the Fe–protein (Ernst et al., 1990a). Other studies have shown that organic carbon (fructose) does not influence the induction of either *in vivo* hydrogen uptake or Nase activities in *A. variabilis*, but exogenous fructose supports higher *in vivo* hydrogen uptake and Nase activities when the cells enter late exponential phase of growth (Troshina et al., 1996).

Table 1−6
Growth of *Anabaena*/*Nostoc* sp. under various conditions in batch cultures.

Strain	*Nostoc* sp.	*Anabaena* sp.	*Anabaena* PCC 7120	*A. circinalis*	*Nostoc* sp.
Medium	BW3	Chu 10	$BG11_o$ (+0.06 g L^{-1} P)	BG11	$BG11_o$(+2 mg L^{-1} Na_2CO_3)
Initial pH	7.70 (+HCl)	——	7.10	——	7.50
T (℃)	30±1	25−32	30	30	30±5
Irradiance	26W m^{-2}	130μmol m^{-2} s^{-1}	120μE m^{-2} s^{-1}	120μE m^{-2} s^{-1}	100μmol m^{-2} s^{-1}
Aeration	——	——	air shaking	2.0%CO_2/air	air
τ (vvm)	0.38	——	——	——	0.2
PBR	10L Mariotte flasks	——	250 mL Erlen	Gas wash bottle	Flat panel
Productivity	0.45 g L^{-1} (7d)	1.25 division d^{-1} (15d)	1.2 g L^{-1} (8d)	1.6 OD_{750nm} (5d)	2.14 g L^{-1} (12d)
Reference	Silva *et al.*, 1994	Nalewajko and Murphy, 2001	Nayak *et al.*, 2013	Hifney, 2013	Johnson *et al.*, 2014

τ = Aeration rate, vvm; Erlen= Erlenmeyer flask; T= Temperature

1.3.2 pH

1.3.2.1 pH affects the growth and metabolism

As one important environmental parameter, culture pH may affect the structure of cell membrane and the activity of some enzymes, and the metabolism of cells will also be impacted.

As reported, the growth of the filamentous cyanobacterium *Nostoc muscorum* is usually best at the pH range of about 7.0 to 8.5, and decreases from pH 7.0 to 6.0, especially toward the lower limit. The buffer used in most of the experiments is K_2HPO_4 – KH_2PO_4. A pH of about 7.2 − 7.4 is optimum, which can be realized by aeration with 1.0 % CO_2 enriched air (Allison et al., 1937). Growth of five *Azolla* populations is found to be comparable from

pH 5.0 to pH 8.0, but decrease is observed at pH 9.0 (Peters et al., 1980). For the strain *A. ambigua* cultivated in BG11 medium, pH 7.0 is also shown to be the best, while alkaline pH (pH 9.0 and 11.0) are proven to be better than at acidic pH (pH 5.0 and 3.0) (Reddy et al., 2013).

It has also been documented that no optimum is observed between pH 5.0 and 9.0 for NH_3 movement through cell wall of *A. azollae* (Zimmerman and Boussiba, 1987). Besides, pH also affects H_2 production by *A. cylindrica* sparged with argon gas in the absence of exogenous Nr (Jeffries et al., 1978). Cultures grown at pH 7.4 produce H_2 at the same initial rates as those at pH 9.4, but the latter cultures can continue to produce H_2 even after CO_2 deprivation.

It has also been investigated that pH of the medium affected the process of light–induced acidification by *A. variabilis*. Known as a rich source of bioactive metabolites, pH is observed to play a role in enhancing the efficiency of *Anabaena* strains as a biocontrol agent (Chaudhary et al., 2013). The pH value also helps to reduce the risk of water contamination caused by cyanobacteria and their metabolites. It is demonstrated that it is critical to keep the water pH above 5.0 at all times in order to make sure of water security (Qian et al., 2014). Three conditions (pH 5.5, 6.0 and 6.5) have significant inhibitory effects on the growth of *A. spiroides* when acidification treatment was conducted during the logarithmic phase (Wang et al., 2011).

At pH 9.0, increase in NH_3 excretion by *Hapalosiphon sp.* and maximum Nase activity have been detected, as well as chlorophyll content, PBP contents, phycobilisome numbers and PSII/PSI ratio. The maximum Nase activity corresponded with dimensions of heterocyts. As it is reported, *Hapalosiphon* sp. is capable to adapt to combined variation of pH and CO_2 at extremely limited irradiance in the range usually found in rice fields (Shokravi and Soltani, 2011).

1.3.2.2 pH affects ammonium uptake and transport

pH also affects ammonium uptake by cyanobacterium *Anacystis nidulans* grown on nitrate. *Anacystis nidulans* possesses two specific ammonium (NH_4^+) uptake systems, both of which have different maximally operative pH, for high-affinity system and low affinity system are separately 7.4 and 5.2 (Kashyap and Singh, 1985). In *Spirulina platensis*, by studying the dependency of NH_4^+ uptake on metabolism (Boussiba, 1989), it has been found that the pH dependency pattern correlating with light-dependent O_2 evolution and dark O_2

consumption. And, the uptake of NH_4^+ is pH dependent with an optimum at pH 9.3 (Boussiba, 1989), but the uptake rates are pH independent over the range 6.2 − 8.7 (Boussiba et al., 1984). In NH_4^+-grown cells of *A. azollae*, no optimum is observed between pH 5.0 and 9.0 for NH_4^+ movement into cells (Zimmerman and Boussiba, 1987). Thus, the effect of pH on NH_4^+ uptake is significantly depending on the species of cyanobacteria.

In a methylamine-resistant mutant strain of *A. variabilis* strain 4m3, the initial rapid and slower second phases of NH_3 uptake show a marked reduction, at pH 7.0 but not at pH 9.0, when compared with the parent strain (Reglinski et al., 1989). High-affinity ammonium transport system in *Nostoc calcicola* is maximally operative at pH 6.0 and the low-affinity system at pH 7.0 (Prasad and Kashyap, 1990).

1.3.3 Light intensity and photobleaching

To a marked extent, the optimum light intensity has been found to depend upon the growth conditions, particularly the medium used, amount of CO_2 or sugar available, and possibly upon the degree of aeration. A considerably lower light intensity is certainly preferable, as some cyanobacteria are sensitive to high light intensities

Nultsch and Agel (1986) have studied the relation between fluence rates and photobleaching in *A. variabilis*, where the PBPs are shown to be more sensitive to high fluence rates than Chl *a* according to the *in vivo* absorption spectra, while the carotenoids are least sensitive (Nultsch and Agel, 1986). After five days of irradiation of very high fluence rates exceeding 50 Wm^{-2} white light, the cells can be photokilled. Photobleaching is a light adaptation process and not simply a photodamage phenomenon, because repigmentation occurs when photobleached cyanobacteria are exposed to low irradiances. Furtherly, the photobleaching of both PBPs and Chl *a* is exclusively caused by wavelengths absorbed by PBPs, mainly phycoerythrocyanin, and red light absorbed by short wavelength chlorophyll (Nultsch and Agel, 1986).

The optimum light intensity for maximum biomass production by *Anabaena* PCC 7120 in flat plate tissue culture flasks under batch cultivation was found to be 120 µmol m^{-2} s^{-1} (Nayak et al., 2013). High light intensity made cultures turn slightly yellow after a few weeks, while the cultures in the lower light remained green. Such results are valid for the

biomass concentrations obtained in their culture system. Indeed higher is the biomass concentration, higher is the self-shading process. Models have been introduced for taking in account the effect of biomass concentration on the light transfer through cyanobacterial populations (He et al., 2015); a basic approach is to introduce the specific light supply rate, as the ratio of the photon flux density, as photosynthetically active radiations, over the biomass concentration.

In addition to light intensity, light/dark cycle plays important role in cell growth and NH_4^+ assimilation by *Anabaena* strains. When the N_2–fixing cyanobacterium *A. variabilis* ATCC 29413 is cultivated under a 12h/12h light-dark cycle in continuous scheme, photosynthetic activity results in a continuous increase in cellular glycogen content in the light, followed by an almost complete dissimilation of the polysaccharide during the dark period (Ernst et al., 1990a). In *Anacystis nidulans* grown on nitrate, two specific NH_4^+ uptake systems are found to be light-dependent, in which energy is more important for NH_4^+ uptake by the low-affinity system, whilst the high-affinity system is involved in proton gradient of membrane (Kashyap and Singh, 1985). NH_4^+ uptake is light independent since it proceeded at the same rates in the light and in the dark in *Spirulina platensis* (Boussiba, 1989). In the two NH_4^+–specific transport systems (high-affinity and low-affinity) of *Nostoc calcicola*, the low–affinity system requires a 2.5−fold higher activation energy than the high-affinity system (Prasad and Kashyap, 1990).

1.3.4 Temperature

1.3.4.1 Effect of temperature on cell growth

Temperature is one of the most significant environmental parameters for cyanobacteria, which can have influence on growth rate, photosynthetic activity, specific respiration rate, etc. With knowledge in this aspect, the control of bloom cyanobacteria or the production of some byproducts will be more controllable. The development of *A. planktonica* blooms is also associated with the increases in surface water temperature, with the peak of the bloom almost synchronous with maximum water temperature (Wood et al., 2010). Under optimal nutrient conditions, *Anabaena* sp. isolated from Lake Biwa grows optimally at 28–32 °C at a maximal growth rate of 1.25 division day^{-1} (Nalewajko and Murphy, 2001). The optimum has not been determined accurately in the growth rate of *Nostoc muscorum*, but over the range of 25 – 30 °C no great difference is observed (Allison et al., 1937). At higher temperatures, especially

at 35 – 40 °C, the growth rate is much slower and the cells, when observed under microscope, frequently appear to be abnormal. At lower temperatures (5 − 10 °C), vegetative cells commonly go into a resting condition as akinetes, in which they will remain dormant for several months. EPS generation by *Anabaena* sp. ATCC 33047 is significantly enhanced in response to an increase in temperature or irradiance (Moreno et al., 1998).

1.3.4.2 Effect of temperature on N_2-fixation and NH_4^+assimilation

Compaoré and Stal (2010) demonstrate that photosynthesis and respiration both contribute to Nase activity in *Anabaena* and *Nostoc* and that their individual contributions depend on both O_2 concentration and temperature (Compaoré and Stal, 2010). Under a 12h/12h light/dark cycle and exposed to different temperatures, Nase activity of both heterocytous cyanobacteria, *A. variabilis* Kützing ATCC29413 and *Nostoc* sp. PCC7120, appear to be the highest at 39 − 42 °C. It is also indicated that 25% and 33% of Nase activity is supported by respiration for *Anabaena* and *Nostoc*, respectively, and the rate increases with increasing temperature. Oppositely, in either organism of heterocytous cyanobacterium *A. cylindrica* and the unicellular cyanobacterium *Gloeothece* sp., N_2 fixation is inhibited at elevated temperatures (37 − 40 °C), which is proven to be a consequence of increased sensitivity to inhibition by O_2, but not due to thermal inactivation of Nase (Gallon et al., 1993). In *Gloeothece*, inhibition of carbon–supported respiratory O_2 consumption by elevated temperature is the probable cause of the increased sensitivity of N_2 fixation to O_2. However, in *A. cylindrica*, thermally induced inactivation of N_2 fixation by O_2 can be correlated with inhibition of hydrogenase.

Study in thermal responses of three physiological processes, N_2 fixation, NH_4^+ assimilation and NH_4^+ uptake in Antarctic and tropical isolates of a diazotrophic cyanobacterium *Anabaena* sp. under influence of various temperatures (5 – 40 °C) has revealed that cyanobacteria can adapt to different temperature levels during long time as well as their physiological processes (Shukla et al., 1997). For example, Antarctic isolate exhibits the above mentioned activities at low temperature (5 °C), but not the tropical isolate. Additionally, the optimum temperatures and energy of activation for all of the above physiological processes differ in both isolates. This study provides base line data for a little–investigated area of the physiological response of Antarctic cyanobacteria under simulated laboratory conditions. This report also explains why different strains perform oppositely at the same condition.

Investigations on temperature profile have revealed that the optimal temperature for the high–affinity NH_4^+ uptake system of *Nostoc calcicola* is 30 °C and for the low-affinity system is 40 °C (Prasad and Kashyap, 1990). Shift-down from 50 °C to 30 °C experiments has proved that irreversible inactivation of the transport process occurred following exposure to critical temperature. A proteinaceous carrier is suggested to be involved in NH_4^+ uptake process based on the inhibition of NH_4^+ transport by higher temperature and chloramphenicol.

1.3.5 Gas

Gas supply has significant influence on the growth of N_2 fixing cyanobacteria, including gas composition (particularly O_2, CO_2 or N_2), and gas flow rate. Here we will have a basic review on the studies about the effect of gas on the growth and metabolism of N_2 fixing cyanobacteria.

Evidently, there seems to be no adequate reason for using air containing more than 1.0 % CO_2 when the strains of *Nostoc* grown at the condition with light intensity sufficiently low and the growth conditions satisfactory (Allison et al., 1937), while aeration with 5.0 % CO_2 makes the growth rate slower. Observations during growth of *Anabaena* sp. PCC 7120, however, have shown that the initial growth with 1.0 % CO_2 is much rapider at higher light intensities (Nayak et al., 2013).

A daily 1.0 % of CO_2 addition in the gas atmosphere can increase H_2 production for 5.8 times, related to the great increase in heterocyts differentiation to approximately 5 times more (Marques et al., 2011). By aerating the cell suspensions of *A. variabilis* ATCC 29413 with a mixture of 99.0% argon and 1.0 % CO_2 for one day under anaerobic induction, the *in vivo* activity of hydrogenase increased approximately 100 times, from 0.02 to 2.0 (μmol H_2 evolved h^{-1} mg^{-1} protein) (Serebriakova et al., 1994). Under an atmosphere of air or N_2, but not argon, PBPs and photosynthesis are restored in the vegetative cells as heterocyts mature and N_2 fixation is initiated (Murry et al., 1984). O_2 was responsible for the loss of Nase in *A. cylindrical* under some conditions (Murry et al., 1984). Mono–fluoroacetate treatment inhibited N_2 fixation only slightly under aerobic and microaerophilic conditions, and the inhibitory effect appears to be at the level of reductant supply. Generation of EPS is markedly enhanced in response to an increase in air flow rate (Moreno et al., 1998).

In a facultatively heterotrophic cyanobacterium *Anabaena* sp., autotrophically grown cells have lost the acetylene reduction activity (ARA) when incubated under anaerobic

conditions; the activity can be maintained in the presence of MSX; or by pretreatment of the cells with a carbon supply (Newton and Cavins, 1985). Heterotrophically grown cells maintained ARA anaerobically in the absence of MSX. Both cell types required light for the maintenance of ARA (Newton and Cavins, 1985). O_2 is ruled out as essential for NH_3 formation (Newton and Cavins, 1985). Reductants needed for Nase are shown to be provided by PSI and PSII in the heterotrophs incubated aerobically in light and MSX is considered to preserve the intracellular pool of reductant. Both autotrophs and heterotrophs are able to liberate NH_3 when treated with MSX under N_2–fixing conditions. DCMU can enhanced NH_3 liberation without affecting ARA (Newton and Cavins, 1985). Aminooxyacetate and aminoacetonitrile are shown to reduce NH_3 release induced by MSX in non–N_2–fixing cultures of *A. cylindrica* (Bergman et al., 1985).

1.3.6 Dilution rate

For continuous cultures, dilution rate is an important affecting factor on the nutrient supply, cell growth state and biomass production. Long time ago**,** *Anabaena flos-aquae* grown in chemostats with phosphate-limiting growth and dilution rate of $0.36 - 0.\mathbf{72}\ d^{-1}$ has already been investigated (Bone, 1971). Induction of heterocyts in *Anabaena* sp. L−31 is totally inhibited by KNO_3 in batch cultures whereas in continuous cultures no inhibition is observed at high dilution rates. When NO_3^- is utilized, NH_3 accumulates in the growth medium, the quantity of extracellular NH_3 declining with increasing dilution rate (Thomas and David, 1971). Nonetheless, **t**he releasing rate of NH_3 per cell increases with decreasing density of organisms, and the induction of heterocyts is consistently observed when NH_3 release per cell exceeds $2 \times 10^{\mathbf{-1}}$ μg. It is inferred that such excessive release depletes the level of intracellular NH_3 causing the induction of heterocyts.

In continuous culture of *Anabaena* sp. ATCC 33047, EPS accumulation in the medium increased in response to a decrease in the dilution rate, with maximal EPS productivity being reached at a dilution rate of $0.72\ d^{-1}$ (Moreno et al., 1998). The relationship between N_2 fixation and NH_4^+ uptake of diazotrophic nanocyanobacteria has been clarified using continuous cultures of *Crocosphaera watsonii* isolated from the western subtropical Pacific (Masuda et al., 2013). Six steady-state growth rates, ranging from 0.10 to $0.35\ d^{\mathbf{-1}}$, have been established under saturating light, corresponding to 20 – 75 % of the maximum growth rate. According to the results, nitrogen fixation do not vary consistently with dilution rate ranging

from 4.4 to 12.9 fmol N cell^{-1} d^{-1}, with the highest rates at intermediate dilution rates. In contrast, ammonium uptake increased significantly with increasing dilution rates over the range of 10 to 80 fmol N cell^{-1} d^{-1} and contributed 65 – 95 % to the daily cellular N requirement. The dissolved organic nitrogen (DON) excretion increased with increasing dilution rate, but only a small portion of assimilated nitrogen can be excreted as DON.

In the evolution process of life on the earth, the role of cyanobacteria cannot be neglected as were the primary organisms with PSII that changed the atmosphere with oxygen and made it possible for the survival of respiratory organisms. With the specific characteristics that fixing both carbon and nitrogen in presence of only some ions in the medium under aerobic conditions by using sunlight as the sole energy resource, they are regarded as in irreplaceable position in the recycle of carbon and nitrogen and also as most potential and useful members in biotechnological application areas, for instance as bio-fertilizer in agriculture, as active compounds producer for food proceeding and medical production, and as wastewater treatment agents for environment protection. Anyhow, they have been paid increasing attention by researchers from a variety of researching fields all over the world for several decades. Sustainable ammonium production is always a challenge, no matter by genetic modifications, from random mutagenesis to multigene transformations. Indeed, extracellular ammonium production by mutants or transformed strains seems the most promising solution as it allows biomass reusing.

1.4 Working hypothesis

The use of photobioreactors makes it possible to control different parameters to obtain reproductible experimental data and to improve the knowledge on the kinetic of extracellular combined nitrogen. Then, informations relevant to some hypothesis could be expected.

1.4.1 A balance equation for the stoichiometry of biomass production

The global balance equation for the stoichiometry of biomass production, assuming there is no extracellular product formation, could be written as:

$$a\mathrm{CO_2} + b\mathrm{H_2O} + \mathrm{cN_2} \rightarrow \mathrm{C}_a\mathrm{H}_{2b}\mathrm{O}_{2a+b-2d}\mathrm{N}_{2c} + d\mathrm{O_2} \qquad \textbf{Eq. 1–1}$$

By using the elemental composition of *A. variabilis* PCC7937 determined by Tsygankov et al. (Tsygankov et al., 1998);

$$CO_2 + 0.845\ H_2O + 0.077\ N_2 \rightarrow CH_{1.69}O_{0.487}N_{0.154} + 1.179\ O_2 \qquad \textbf{Eq. 1–2}$$

The stoichiometry shows that for one mole biomass 0.077 mol of N_2 have to be fixed, or 0.11 g NH_3 per g of dry biomass. For instance, the biomass productivity of 10 g m^{-2} d^{-1} reported in free cell cultures of *Anabaena*, could correspond to 0.9 g N_2 m^{-2} d^{-1} fixed (Moreno et al., 2003).

> ***Hypothesis 1****: an estimation of the rate of dinitrogen fixation could be obtained through the rate of dry biomass production in photobioreactors.*

1.4.2 Speciation of combined nitrogen in the culture medium

As previously shown, a production of extracellular combined nitrogen could be induced in diazotrophic cyanobacteria. It consists to partially inhibit the glutamine synthetase activity, either through chemical treatment with MSX or through genetic transformation (Bui et al., 2014). It was shown that significant amounts of ammonium could be recovered in the culture medium (Park et al., 1991 ; Paschkewitz and Leddy, 2012). However, there has been no report on the repartition of both nitrogen forms as a function of the pH of cyanobacterial cultures. Indeed under normal diazotrophic conditions, the assimilation of dinitrogen is not associated to pH variations, by contrast with nitrate or ammonium assimilations respectively associated to increase and decrease of pH, as a consequence of OH^- and H^+ exchanges (Bui et al., 2014). In previous publications, authors were working with global ammonium concentrations, including NH_3 (Newton and Cavins, 1985 ; Paschkewitz and Leddy, 2012). Indeed N_2 exchange between the culture medium and the heterocyt is by diffusion.

Moreover, the pH of the culture was generally not controlled. The absence of pH variation under diazotrophic metabolism is easily assessed when cyanobacteria are cultivated in controlled photobioreactors. The nitrogen fixation is usually measured through the

acetylene reduction assay (ARA). Another way is to deduce the rate of nitrogen fixation through the mass balance of nitrogen from the data on biomass productivity.

When the GS activity is partially blocked, this stoichiometric equation could be written as:

$$a\mathrm{CO_2} + b\mathrm{H_2O} + \mathrm{cN_2} \rightarrow \mathrm{C}_a\mathrm{H}_{2b-3e}\mathrm{O}_{2a+b-2d}\mathrm{N}_{2c-e} + d\mathrm{O_2} + e\mathrm{NH_3} \qquad \textbf{Eq. 1–3}$$

The NH_3 produced through the nitrogenase activity within the heterocyt is assumed to obey to its dissociation constant under the control of the intracellular pH;

$$\mathrm{NH_3 + H_2O} \leftrightarrow \mathrm{NH_4^+ + OH^-} \qquad \textbf{Eq. 1–4}$$

According to the **Eq. 5**, the acid dissociation constant pK_a is 9.01 at 30 °C. Then, the percentage of NH_3/NH_4^+ expected to be excreted in the culture medium is also a function of pH as calculated according to this equation (Körner et al., 2001).

$$\mathrm{pK_a} = \frac{0.09108+2729.92}{(273.2+T)} \qquad \textbf{Eq. 1–5}$$

$$\mathrm{pK_a} = \mathrm{pH} + \log\frac{[NH_4^+]}{[NH_3]} \qquad \textbf{Eq. 1–6}$$

Then, undissociated ammonia fraction is calculated as:

$$\mathrm{f} = \frac{1}{(10^{pKa-pH}+1)} \qquad \textbf{Eq. 1–7}$$

Data on the intracellular pH, when GS activity is inhibited, are not available. Experimentally, as cultures of heterocytous cyanobacteria were cultivated for long terms with MSX, it could be assumed that the pH increase within cells, as expected from Eq. 3, could be metabolically controlled. Nowadays, the secretion process of NH_3/NH_4^+ remains to be clarified (Böhme, 1998; Sah, 2008). Indeed, it could involve either a diffusion of process of ammonia, or an ammonium transporter (Amt) (Khademi, 2004 ; Soupene et al., 2002).

If the excretion involves ammonia as the main form of reactive nitrogen, then we must expect a pH increase associated with the kinetic of incubation in the presence of MSX. In the

other case, the excreted ammonium would act as a weak acid in the culture medium as it dissociates in ammonia and hydrogen ions as:

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad \textbf{Eq. 1–8}$$

***Hypothesis 2**: a pH variation in unbuffered cultures of MSX treated cyanobacteria could be an indication of the process involved in the combined nitrogen transfer between heterocyts and the culture medium.*

Indeed the repartition of NH_4^+ /NH_3 in the culture medium is also dependent on the pH. Then, if the accumulation of reactive nitrogen is associated to pH change, it could be expected progressive changes in its speciation.

For instance,

At pH 6.8, $[NH_3]_{aq}$ = 0.5 % $[NH_4^+ /NH_3]_{aq}$,

At pH 8.8, $[NH_3]_{aq}$ = 33.6 % $[NH_4^+ /NH_3]_{aq}$,

***Hypothesis 3**: illustrated in **Figure 1–9**, we can expect that the pH variations could be related to the total NH_4^+/NH_3 production rate.*

1.4.3 Solubility of ammonia gas

The solubility of ammonia gas in water is high (Dasgupta and Dong, 1986). The equilibrium $[NH_3]_{aq} \leftrightarrow [NH_3]_g$ is described by the Henry's law $H^{cc}= \frac{[NH_3]aq}{[NH_3]g}$. For ammonia, the relation $Lg_{10}\ H^{cc} = \frac{1477.7}{T} - 1.694$ was proposed by Hales and Drewes (1979) for the partition between air and water (Hales and Drewes, 1979):

Then, at 30°C H^{cc} is 1515.25

Then, as *Anabaena* is cultivated at 30°C in the $BG11_o$ culture medium, with a salinity lower than 1 g L^{-1}, the NH_3 solubility is about 400 g kg^{-1}. By contrast, the concentrations of NH_4^+/NH_3 excreted by cyanobacteria are generally lower than 400 mg kg^{-1} (Körner et al., 2001). Indeed, at concentrations higher than 10 mg L^{-1}, ammonia is known to be toxic to microalgae and cyanobacteria (Körner et al., 2001).

***Hypothesis 4**: it could be expected that one of the limitation for ammonia excretion by cyanobacteria could be an auto-inhibition effect.*

1.4.4 Gas exchanges within cultures of diazotrophic cyanobacteria

The cultivation of cyanobacteria in photobioreactors is associated to gas transfer in order to provide CO_2 and to remove excess O_2 to/from cultures, respectively carbonatation and deoxygenation.

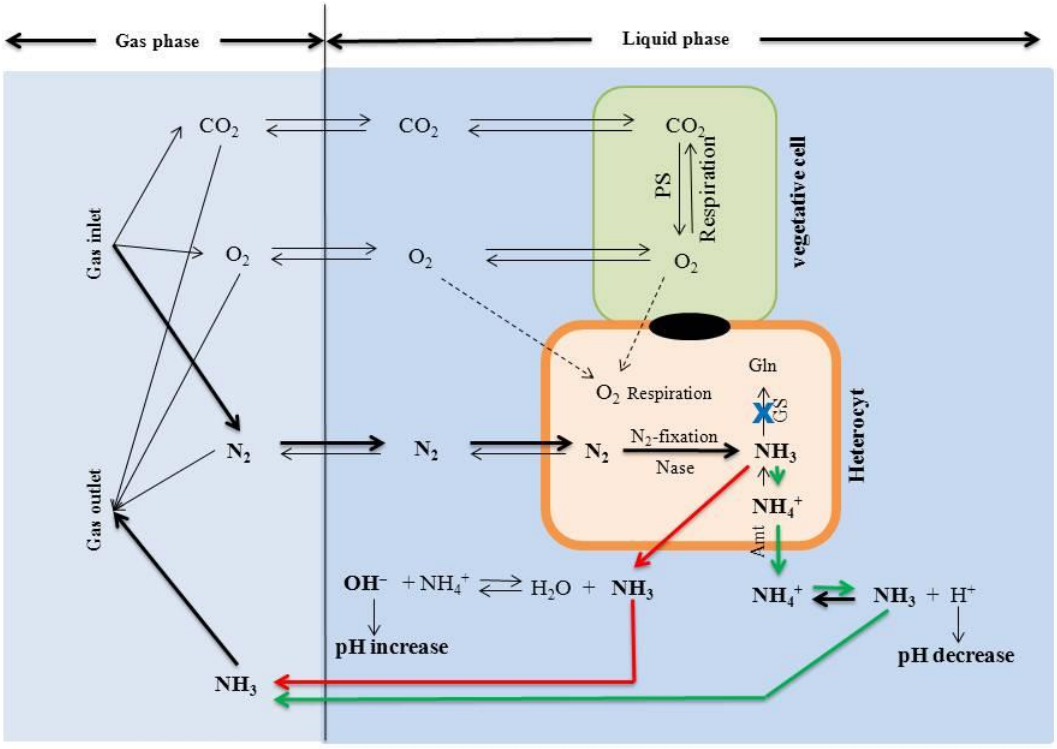


Figure 1−9. Gas transfer among heterocytous cyanobacteria *A. variabilis* cells, culture medium and the gas phases ; (adapted from Walsby, 2007).

Then, we have to take in account the gas/liquid transfers within the photobioreactor, which are reactions at desequilibrium, as the ammonia is assumed to be continuously produced in the culture medium by the cyanobacteria. The two-film theory of gas transfer is the best way to describe the gas/liquid transfer process (Lewis and Whitman, 1924). Accordingly, at the interface of the two phases there is a limitation layer, whose thickness depends of the hydrodynamic (mixing) and of some characteristics of the gas and liquid, such as viscosity.

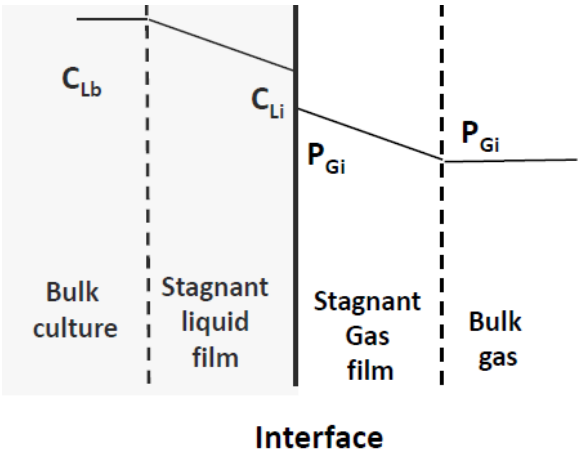


Figure 1−10. Ammonia gas transfer in the photobioreactors according to the two-film theory of gas/liquid transfer (Lewis and Whitman, 1924).

The efficiency of the gas/liquid transfer within the photobioreactor could be determined through the k_La value as described in the next equation:

$$\frac{d[NH_3]}{dt} = k_La\,([NH_3]_{aq} - [NH_3]_{aq*}) + X \times q[NH_3] \qquad \textbf{Eq. 1−9}$$

where, $q[NH_3]$ is the specific rate of ammonia production;

X is the biomass concentration

$[NH_3]_{aq*}$ dissolved ammonia concentration at equilibrium with the partial pressure of ammonia in the gas phase ;

The air used for culturing microalgae and cyanobacteria are generally devoid of ammonia.

***Hypothesis 5**: increasing gas transfer could improve the rate of combined nitrogen production by displacement of the equilibrium of the simplified reaction.*

$$N_2 + Biomass \rightarrow Biomass + [NH_4]^+ + [NH_3]_{aq} \leftrightarrow [NH_3]_g$$

1.4.5 Gas exchanges within an acid trap

Different strategies have been studied in order to valorize the ammonia/ammonium produced by heterocytous cyanobacteria. The most studied one is the cultivation of immobilized cyanobacteria, mainly by encapsulation within calcium alginate beads. By this way the culture medium is progressively enriched in combined nitrogen for further use as bio-fertilizer (Jeanfils and Loudeche, 1986 ; Musgrave et al., 1982). Co-cultures of the diazotrophic bacteria *Azotobacter* with *Chlorella* have also been studied (Lipman and Teakle, 1925), but there are no published data on the potential use of a gas stripping process for energy efficient ammonia recovery.

The rate of ammonia stripping could be described based on the ammonia mass transfer coefficient for the culture medium (k_La, NH_3), assuming NH_3 in not present in the sparger gas input of the photobioreactor. Then, the diffusion of NH_3 to the gas phase can be estimated from the balance between the gas and the liquid phases assuming a pseudo-steady state.

In a photobioreactor, the k_La values are known to depend on the gas flow rate, expressed as vvm, the ratio of the gas flow rate (e.g. L min^{-1}) on the culture volume (e.g. L) (Zhang et al., 2002).

Hypothesis 6*: varying the gasing rate of the photobioreactor is an easy way for determining the impact of k_La on the potential ammonia stripping from the photobioreactor.*

Using an efficient acid trap is a reliable method for a quantitative estimation of the mass of NH_3 stripped from chemical or biological reactors (Ndegwa et al., 2009). Then, a set-up composed of a photobioreactor associated with an acid trap could be a good way to check the hypothesis of ammonia production.

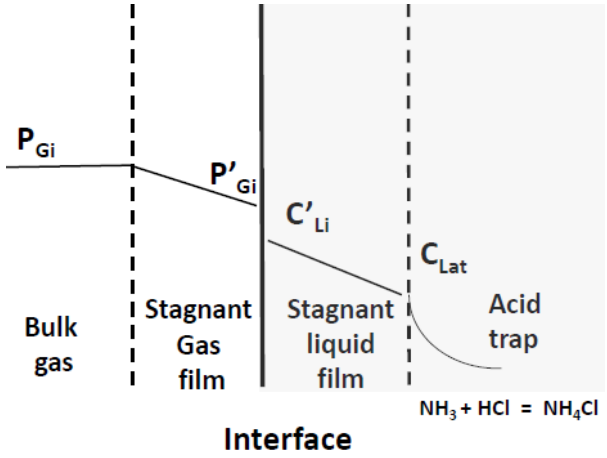


Figure 1−11. Ammonia trapping ; ammonia transfer from gas phase to the HCl solution.

If the amount of acid in the trap is in excess, then all the ammonia will be neutralized and accumulated as ammonium. Indeed the rate of such proton transfer reaction is very high, estimated at 1×10^{9} mol L^{-1} s^{-1}, as it is the reaction between a strong acid and a weak base. Then under the assumption that the concentration of ammonia in the acid trap tends to zero, the transfer only depends on the partial pressure of ammonia in the gas phase and the k_La in the acid trap and the Henry's law coefficient. The ammonia amounts accumulated in the acid traps could be used to demonstrate the stripping effect and to measure its efficiency.

The accumulating rate of ammonia in acid solution makes it possible to manage the balance of gas ammonia production in the photobioreactor.

CHAPTER II Materials and methods

2.1 Strain and medium

2.1.1 Strain

The wild type strain of cyanobacterium *Anabaena variabilis* Kützing PCC 7937 used in this work is from Pasteur Culture Collection of Cyanobacteria (PCC) (Rippka and Herdman, 1992) ; it is available as PCC 7937 = ATCC 29413 (Komárek et al., 2014). This cyanoprocaryote is identified as *Trichormus variabilis* (Kützing ex Bornet et Flahaut) Komárek and Anagnostidis (Komárek and Anagnostidis, 1989) and sometimes unduly identified as *Nostoc*. This filamentous heterocytous cyanobacterium can fix dinitrogen to ammonia but cannot release ammonia out of cells (**Figure 2−1**).

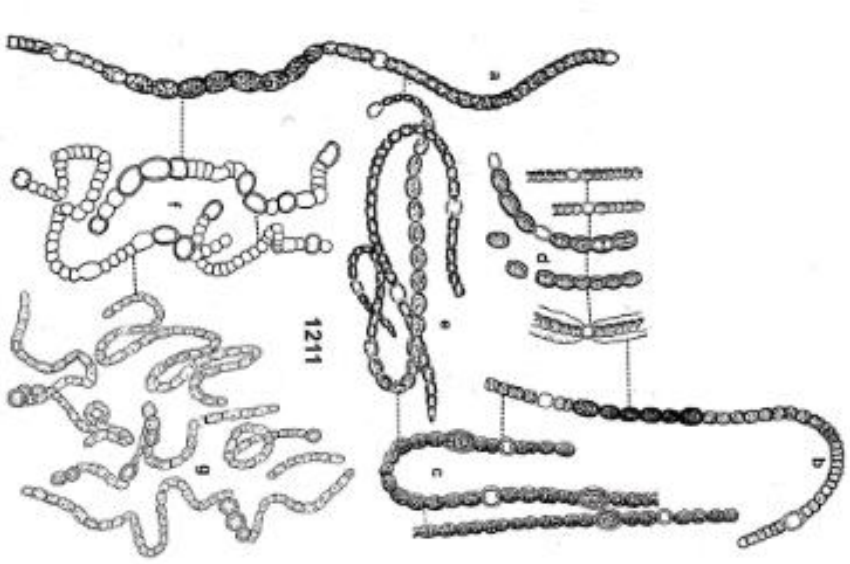


Figure 2−1. *Anabaena variabilis* Kützing, a : adapted from Frémy ; b : adapted from Watanabe, 1971 ; c : adapted from Berger et al. 2005 ; d : adapted from Obuchova 1959 ; e : adapted from Setchek & Gardner 1919 ; f : adapted from Prescott ; g : adapted from El-Ayouty et al. 1977 ; adapted from Komárek, 2013 ; Geitler, 1932.

The scientific classification:

Empire	Prokaryota
Kingdom	Eubacteria
Phylum	Cyanobacteria (Cyanoprokaryotes)
Class	Cyanophyceae
Order	Nostocales
Family	Nostocaceae
Genus	*Anabaena*
Species	*Anabaena variabilis* Kützing

The mutant strain *A. variabilis* PCC 7937-C9 was isolated by a bi-step mutagenesis (Bui et al., 2014). This strain has been proven to have a good stability that it has conserved an ammonium excreting activity in presence of MSX over more than 4 years.

The wild type (WT) and mutant strain (C9) were maintained on $BG11_o$ (+ 1.5 % agar) agar plates for long term. At the same time, they were also kept in 250 mL Erlenmeyer flasks containing 100 mL $BG11_o$ medium in an orbital incubator shaker at 100 rpm, temperature of 30 °C and a continuous irradiance of 50 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent tube lamps, which were sub–cultured every one to two weeks. Pre–cultures for photobioreactors were grown in a 300 mL column. The subcultures were regularly verified for absence of bacteria and fungi by control on Plate Count Agar (Fluka).

2.1.2 Culture medium

The BG11 medium is widely used for the cultivation of various microalgae, such as *Anabaena*, *Nostoc*, (Johnson et al., 2014 ; Lehner et al., 2013; Shi et al., 1987). According to the purpose, it is sometimes with combined nitrogen while sometimes without nitrogen. In our study, ammonium/ammonia production which come from nitrogen fixation is the target, so all the medium used were without combined nitrogen, namely $BG11_o$ medium.

The composition of $BG11_o$ medium, natural pH (Rippka et al., 1979), (per liter) was 0.04 g $K_2HPO_4 \cdot 3H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.036 g $CaCl_2 \cdot 2H_2O$, 1.2 mg Na Fe EDTA, and 1.0 mL trace metal solution A5. Composition of trace metal solution Stock 5 (per liter) was 2.86 g H_3BO_3, 1.81g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 0.05g $CoCl_2 \cdot 6H_2O$, and 0.391 g $Na_2MoO_4 \cdot 2H_2O$ (Allen and Stanier, 1968).

Table 2−1
Composition of the stocks for preparing $BG11_o$ medium.

Stock	Chemical	Concentration (g L^{-1})
Stock 1	Na Fe EDTA	1.2
Stock 2	$CaCl_2 \cdot 2H_2O$	3.6
Stock 3	$MgSO_4 \cdot 7H_2O$	7.5
Stock 4	K_2HPO_4	3.05

Stock 5 (microelements)	H_3BO_3	2.86
	$MnCl_2 \cdot 4H_2O$	1.81
	$ZnSO_4 \cdot 7H_2O$	0.222
	$CuSO_4 \cdot 5H_2O$	0.079
	$CoCl_2 \cdot 6H_2O$	0.050
	$NaMoO_4 \cdot 2H_2O$	0.391

The basic $BG11_o$ medium combines the following stock solutions:

Stock solution	Per liter of medium
Stock 1	1 mL
Stock 2	10 mL
Stock 3	10 mL
Stock 4	10 mL
Stock 5	1 mL

Combine the stocks following: stock 1 – 2 – 3 – 5 – 4, stock 4 should be sterilized separately and added to the medium after cooling, to prevent precipitation (Allen and Arnon, 1955). The medium was sterilized at 121 °C for 25 min. All the stocks were stored at 4 °C in the fridge. For continuous cultures in chemostat, $BG11_o$ (2P) medium was used which contained twice amounts of stock 4.

Solid $BG11_o$ medium was used for preservation of strains. 15 g L^{-1} agar was added to $BG11_o$ liquid medium and autoclaved at 121 °C for 25 min. Then, 10 mL medium were added to each Petri dish. 0.5 mL cultures from Erlenmeyer flasks were inoculated on the agar plates. Since strong irradiance would damage the cells on agar plates, a piece of thin paper was needed to cover the agar plates at the beginning. These agar plates were kept at room temperature and with weak irradiance in the Strain Collection in the laboratory.

2.2 Culture system and experimental set-up

2.2.1 Batch culture for inoculum

The batch cultivation was carried out in 300 mL columns containing 200 mL $BG11_o$ medium. Aeration was provided by injecting 0.2 μm filtered air to the bottom of the columns at a rate of 0.2 vvm (volume per volume per minute). These columns were inoculated with cultures from Erlenmeyer flasks, which were in their log phase of growth (0.05 g L^{-1} dry

weight). These inoculated columns were placed in a water bath for maintaining a temperature of 30 ± 2 ^{o}C, which received illumination by fluorescent lamps from one side. The light intensity was adjusted to the required levels, normally 50 µmol m^{-2} s^{-1} as Photosynthetic Photon Flux Density (PPFD), corresponding to Photosynthetically Active Radiations (PAR), by changing the number of cool-white fluorescent lamps and their distances. Light intensity was measured at the surface of the columns with a quantum sensor (Li–Cor, Model LI–250A, USA) and light meter (Li–Cor, Model LI-250A, Lambda Instrument Corp., USA).

2.2.2 Continuous culture for biomass improvement

Experiment design

The design of experiments for the continuous cultures in airlift photobioreactors is presented in the **Figure 2−2**. Experiments were conducted with the wild type (WT) and the mutant strain (C9) of *A. variabilis* PCC 7937. The modified $BG11_o$ medium was used for preliminary cultivations. Indeed, for continuous experiments, the feeding medium was $BG11_o$ (2P) in order to avoid phosphate limitation observed during preliminary batch cultures and at low dilution rates with CO_2 addition. The first run was with the wild type (WT) at 11 dilution rates, and the second run was done with the mutant strain (C9) at 5 relevant dilution rates.

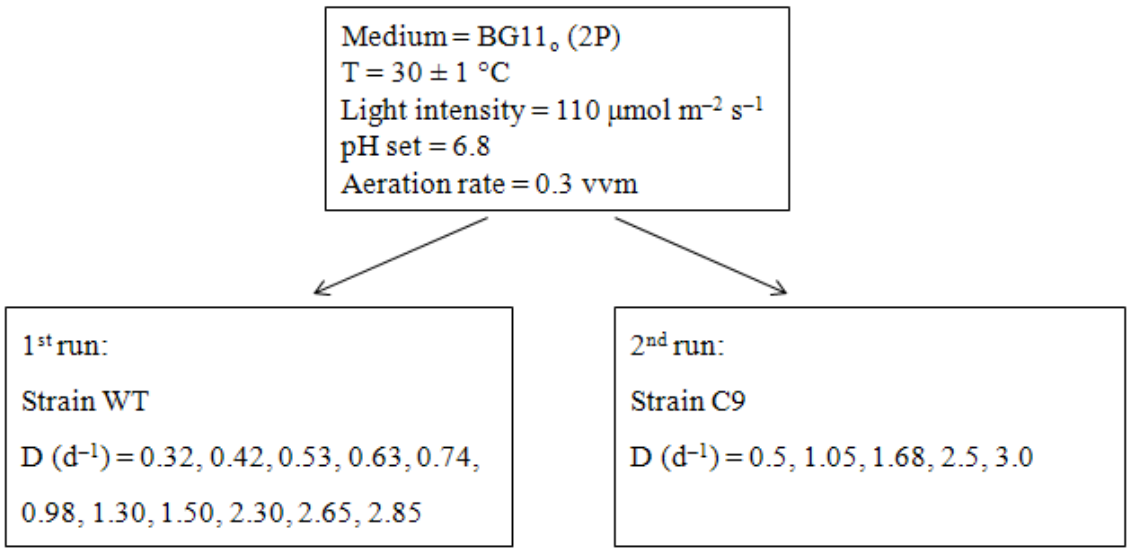


Figure 2−2. Experiment design of chemostat cultures in airlift photobioreactor at different dilution rates.

Cultures were illuminated continuously at a photon flux density of 110 µmol photons m^{-2} s^{-1} in the 400-700 nm wavelengths (photosynthetically active radiation, PAR). The pH was controlled by automatic CO_2 injection (pH−controller Mettler Toledo M200) for value set at 6.8, after reaching a steady−state at the operating pH, mean duration of 10 days.

pH oscillations for a pH control loop

The key point in this part of experiments is the control of pH. When *A. variabilis* strains are cultivated under diazotrophic condiditons with $BG11_o$ medium (pH=6.8 ± 0.2), no pH variation occurs in the culture. In our pre-experiment, it was observed that supplying CO_2 to the culture could significantly increase the biomass production but the pH decreased sharply from 7.0 to 4.0, deleterious to the cyanobacterial cultures. The technical question was to find a way to supply CO_2 to the cultures without using the classical control loop process associated to pH variations of the cultures.

A preliminary study has shown that no significant biomass improvement was achieved by CO_2 of the feeding culture medium (15 L, in a tank well closed).

Then a practical mean for CO_2 addition mixed with air was to fix a set value of pH at 6.8, since the natural pH of $BG11_o$ (2P) medium is 7.2−7.3. The LabView software was programmed with a set pH value of 6.8 and an open time of 2 seconds over 100 seconds for the CO_2 valve.

In this way, culture pH was successfully controlled at 6.8±0.5, but with an oscillation between 6.8 and 7.2. Then, CO_2 was regularly supplied by using a controlled oscillation of the pH values to improve the biomass production.

Experimental set-up

Figure 2−3 presents the set-up of the airlift photobioreactor (PBR). The PBR is made of glass with screw with an insert for a pH sensor. The working volume (V) was 0.7 L.

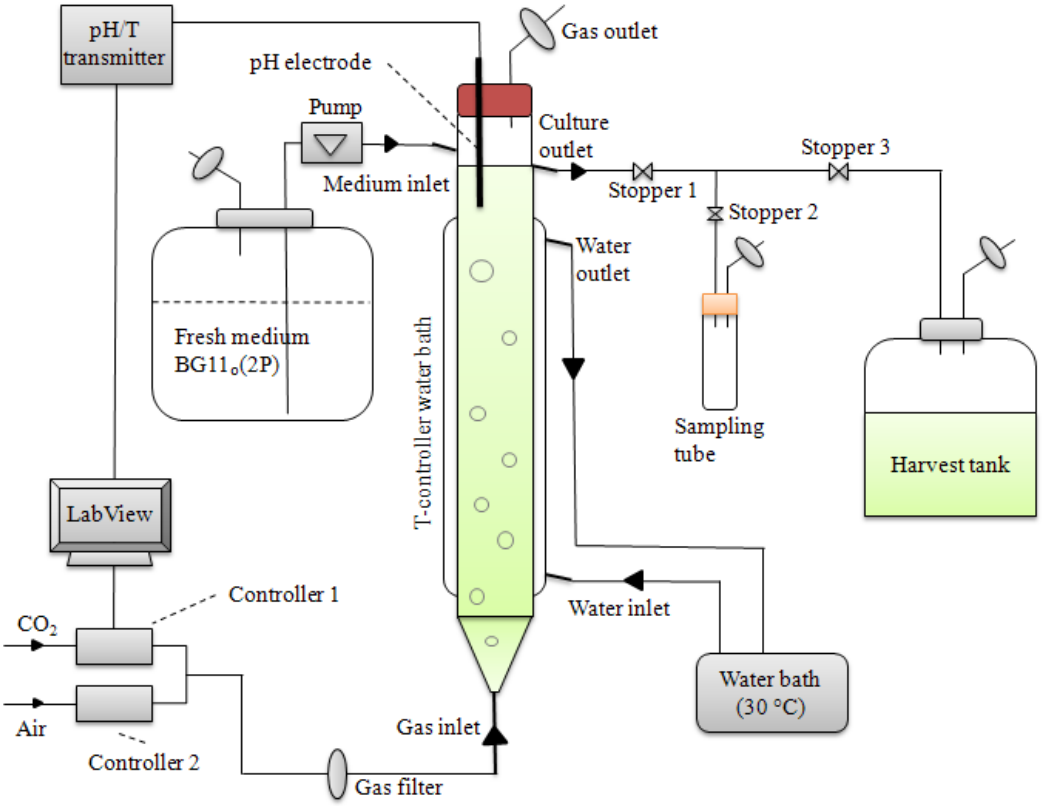


Figure 2−3. Scheme of set-up for biomass improvement of *A. variabilis* PCC 7937 strain.

The scheme of the setup for biomass improvement is as shown above. Sampling tube and harvest bottle were autoclaved (121 ºC, 25 min) before connected. To sample a volume Vs, stopper 1 is closed for a period corresponding to Vs/F (F = liquid flow rate) to get higher level of culture inside the PBR; stopper 1 and stopper 2 will be open and stopper 3 will be closed when sampling, while stopper 1 and stopper 3 will be open and stopper 2 closed at other times. The pH was controlled by automatic CO_2 injection (Controller 1; pH-controller Mettler Toledo M200) for set value pH 6.8; Controller 2 (air flow controller) is open all the time at constant air flow rate, 210 mL min^{-1}, corresponding to an aeration rate of 0.3 vvm. All injected gas was passed through a bacterial gas filter with 0.2 µm pore size (Minisart SRP–25, Sartorius, USA) prior to injection.

Temperature and pH were monitored with sensor (Mettler Toledo SG 3253). Temperature was controlled at 30 ± 2 ºC with water bath, from which the cooling water was circulated through a water-jacket surrounding the bubble column.

Four white fluorescence lamps were used to isotropically illuminate the PBR. The mean value was the average of measurements made at eight different locations on the surface

of the reactor. The initial light intensity without media and culture was used to quantify the light intensity. The incident light area S was 0.06 m^2 and then a S/V ratio of 85 m^{-1}.

The PBR was inoculated with 0.3 L inoculum and supplemented with fresh culture medium $BG11_o$ (2P) to fulfill the PBR, and was operated in batch mode for 5 days and then transitioned to continuous mode by supplying a constant infusion of fresh, sterile media, and withdrawal of culture at the equal volumetric rate as the fresh medium infusion. Once a steady state was reached, measurements at the state were done during four or five days. Later, the flow rates were modified and the reactor was allowed to attain a new steady state.

The chemostat cultures were fed with $BG11_o$ (2P) medium at different dilution rates. (d^{-1}) calculated by the following relation:

$$D = \frac{F}{V_w}$$

where,

F is the medium feeding rate (L d^{-1}),

V_w is the working volume (L).

In continuous culture, the biomass concentration in PBR (X), the specific growth rate (μ) of microalga and the dilution rate (D) are related to the equation as flows:

$$\frac{dX}{dt} = \mu \cdot X - D \cdot X$$

Then, at steady state,

$$\mu = D$$

2.2.3 Batch cultures for kinetics analysis of NH_3 excretion

Experimental set-up

A. variabilis PCC 7937-C9 is a mutant strain of *A. variabilis* PCC 7937 obtained by Lan Anh BUI (Bui et al., 2014). In this study, all cultures came from continuous cultures at steady state cultivated under diazotrophic conditions in 0.7 L cylindrical airlift reactors at a dilution rate of 0.3 d^{-1} with a biomass concentration of 0.3 g L^{-1}. These cells were cultivated at 30±1 °C, successively illuminated with fluorescent lamps at a PFD of 110 μmol m^{-2} s^{-1}, bubbling with air at a flow rate around 0.3 vvm.

For the assays carried out in mini-PBRs, air was injected and main environmental parameters were controlled as needed, including PFD, T, Fg and C_{MSX} (**Figure 2−4**).

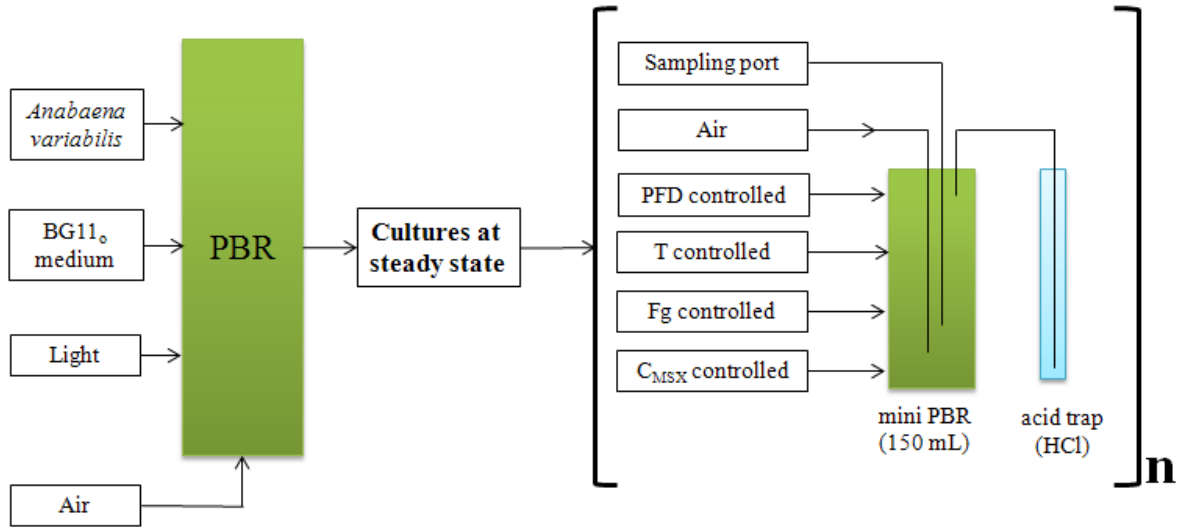


Figure 2−4. Flow diagram of the setup for the study of NH_4^+/NH_3 production in short term experiments. PBR=Photobioreactor; PFD=Photo Flux Density, µmol m^{-2} s^{-1}; T=Temperature, °C; Fg=Gas flow rate (Fg will be transferred to τ, aeration rate), vvm; C_{MSX}=MSX concentration, µmol L^{-1}; n=the number of mini PBR for each assay, up to 8.

NH_3 excretion assays in batch culture for short term were carried out within a set of mini PBRs (**Figure 2−5)**. A mini-PBR is a glass column with a total volume of 160 mL (working volume 100 mL). It is sealed with a silicon stopper, through which a tube was used to provide aeration and a syringe of 1.0 mL with long plastic tube for sampling. The gas outlet was connected to another tube containing an HCl solution (pH 1.5) for trapping the part of NH_3 released to gas phase (**Figure 2−5** and **2−6**).

For NH_3 excretion assays, light was supplied continuously with fluorescent lamps that were placed on one side of the mini-PBRs. The light intensity was adjusted to various levels by changing either the distance from fluorescent lamps to the PBRs or the number of fluorescent lamps. The standard light intensity was 100 − 110 µmol m^{-2} s^{-1} unless in the assays of PFD effect. Temperature was controlled with a water bath by a temperature controller (Eheim Jager, 3611 Aquarium Heater, 25 W, 220 − 240 V, Germany). Gas flow rates (Fg) were adjusted one by one at the beginning of each assay using a portable mass flowmeter (Digital flowmeter, Varian, Analytical Instrument), and transferred to aeration rate (τ) according to the following relation:

$$\tau = \frac{F_g}{V_{culture}}$$

where,

Fg is the gas flow rate, L min^{-1},

$V_{culture}$ is the volume of culture, L.

Light intensity was measured inside PBRs when they were empty and in the working position by a quantum sensor (Li−Cor, Model LI−250A, USA) and light meter (Li-Cor, Model LI−250A, Lambda Instrument Corp., USA).

A stock solution of 5.0 mmol L^{-1} MSX was sterilized by filter (Minisart, 16534-K CE 0120, Sartorius Stedim Biotech GmbH, Germany); 1 mL L^{-1} was added to the inoculum in PBRs as needed. During the whole process of test, the stoppers were never opened in case of NH_3 (gas) loss, but the air bubbling was stopped when sampling, to avoid NH_3 loss.

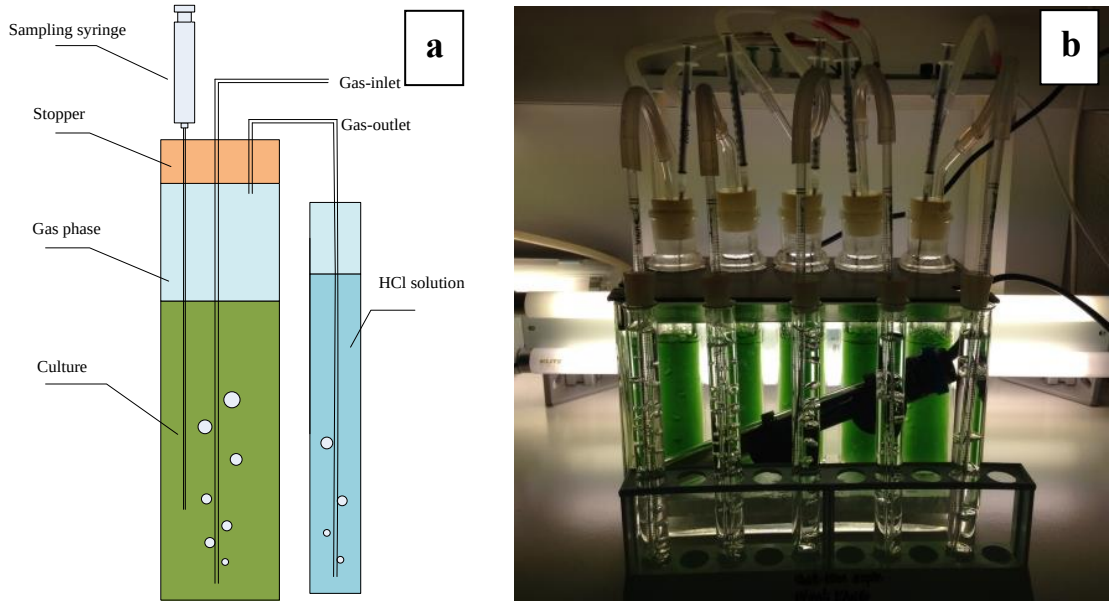


Figure 2−5. Mini-Photobioreactor (PBR) for studying kinetics of NH_3 excretion by *A. variabilis* PCC 7937-C9 in the presence of MSX with HCl stripping solution (**a** configuration; **b**, physical view).

Since various environmental parameters were investigated in NH_3 excretion assays in batch cultures during short terms, such as temperature, irradiance, MSX concentration, gas flow rate and so on, all these parameters were set as needed in different assays.

Design of experiments

Figure 2−6 presents the design of experiments for the short-term studies. Measurements were done each 2 or 3 hours according to the experimental purpose and

necessity. Gas bubbling was stopped by closing the main switch. Samples of HCl stripping solution was taken at small volume and kept in 1.5 mL Eppendorf tubes with caps closed in case of evaporation of NH_3, such as 0.1 mL or 0.05 mL according to the pre−experiments. Each sample of the cultures was 2 mL, which was put in a 15 mL centrifuge tube for pH determination immediately after sampling. Then, 1.8 mL of each was transferred to a 2 mL Eppendorf tube for centrifugation. A certain volume of supernatant was taken for $[NH_4^+/NH_3]$aq measurement, and the remained part of supernatant was put in another Eppendorf and kept for P/I curve determination together with the cells remained in the 15 mL centrifuge tube.

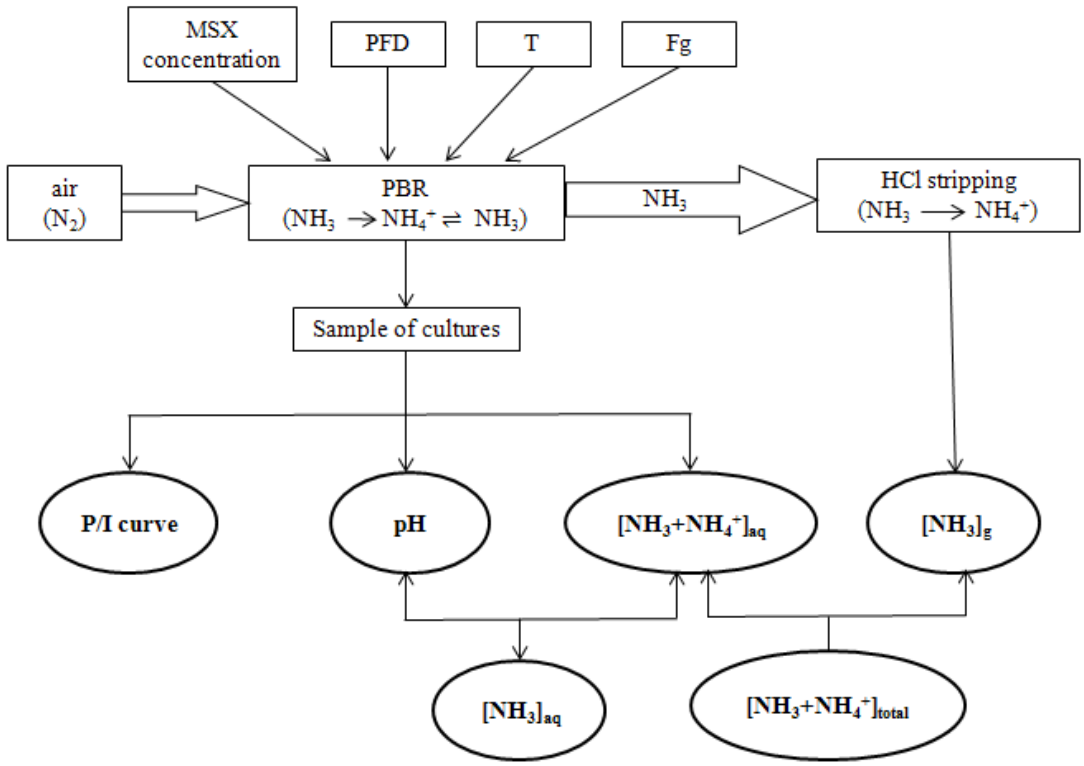


Figure 2−6. Global protocol for the determination of the relevant parameters; pH; P/I curve = photosynthesis-irradiance curve; $[NH_4^+/NH_3]$aq, $[NH_3]$aq, $[NH_3]$g and $[NH_4^+/NH_3]$ total).

It is important to note that, in this study, "$[NH_4^+/NH_3]$aq" refers to the sum of both forms of extracellular ammonium ions (NH_4^+) and ammonia molecules (NH_3) maintained in the culture as transformation between NH_4^+ and NH_3 exists in solutions (Thurston et al., 1979), while "$[NH_3]$g" refers to the part of NH_3 which liberated out of the culture and stripped in HCl solution.

2.2.4 Continuous culture for kinetics of NH_3 excretion

Design of experiments

The experiments for NH_4^+/NH_3 excretion kinetics by *A. variabilis* PCC7937-C9 in continuous culture with pH regulation was designed as shown in **Figure 2−7**. The basic condition was selected according to the biomass improvement in function of dilution rate and NH_4^+/NH_3 excretion studies in batch culture. For the investigation of NH_4^+/NH_3 excretion in batch cultures, the initial biomass concentration was 0.3 g L^{-1}. For the mutant strain C9, the maximum biomass concentration obtained in our study was around 0.6 g L^{-1}. Then, the condition under which the biomass was maximal was selected for the study of NH_4^+/NH_3 excretion kinetics in continuous culture.

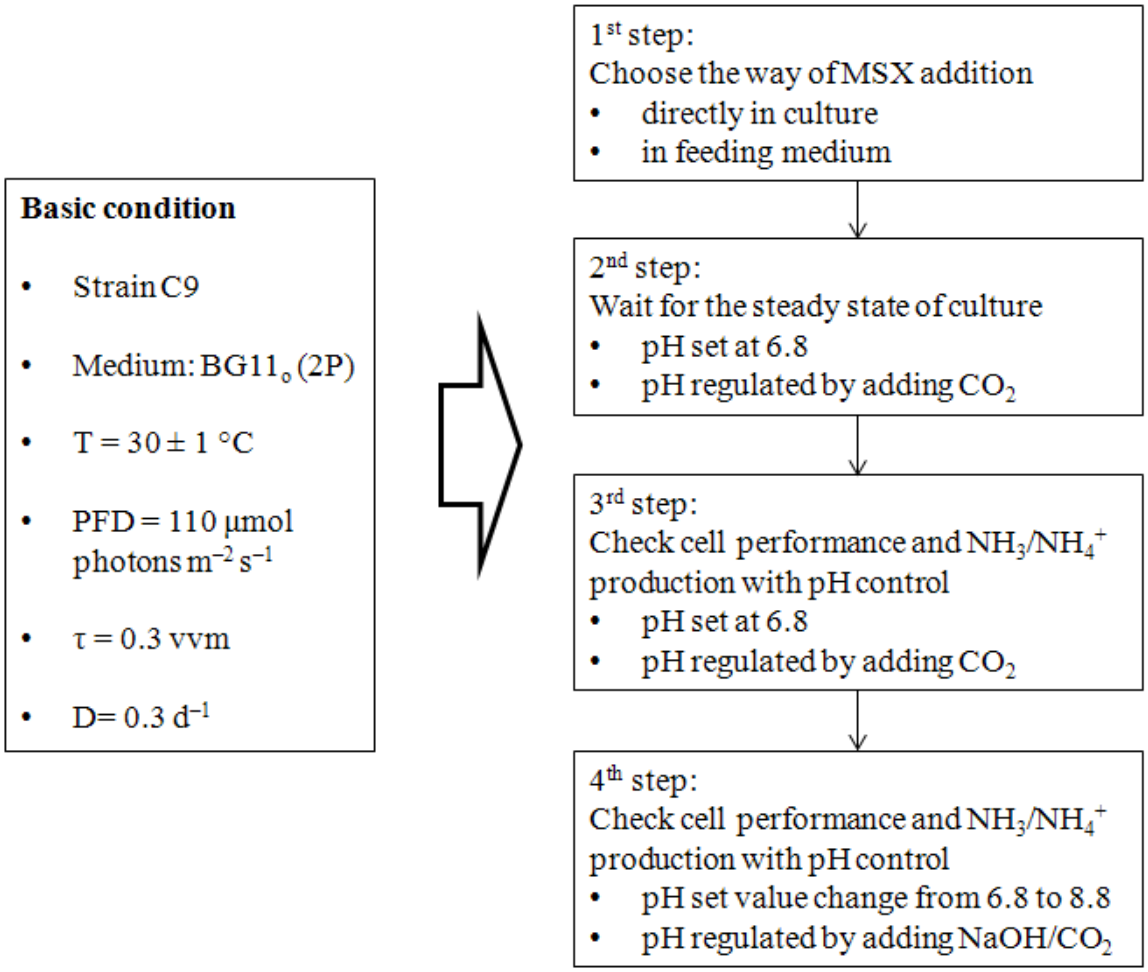


Figure 2−7. Design of the experiments of NH_4^+/NH_3 excretion kinetics by *A. variabilis* PCC7937-C9 in continuous culture with pH regulation.

The first thing was to find a way to add MSX to the continuous culture. Two ways were compared, directly add MSX to the culture and to the feeding medium. Then, at pH 6.8, the performance and NH_4^+/NH_3 excretion kinetics were investigated. By increasing pH to 8.8, NH_4^+/NH_3 excretion kinetics in continuous culture was analyzed to see if it can improve the production of NH_4^+ and NH_3.

Experimental set-up

Another photobioreactor was used for the study of kinetics of NH_4^+/NH_3 excretion by *A. variabilis* PCC7937-C9 in continuous culture. This PBR is the same used for improving biomass productivity, with pH regulation, (**Figure 2−8)** with the same pH regulation system as described in **Figure 2−3**. The working volume is 0.7 L. Fresh medium $BG11_o$ (2P) was continuously fed to the PBR at a dilution rate of 0.3 d^{-1}. Air flow rate was 300 mL min^{-1}, equaling to 0.3 vvm. CO_2 was automatically injected depending on pH value. Two pH values were set, 6.8 and 8.8 during whole process. The temperature was controlled at 30±2 °C, and light was supplied with four lamps. The difference is that this PBR was connected to an acid trap.

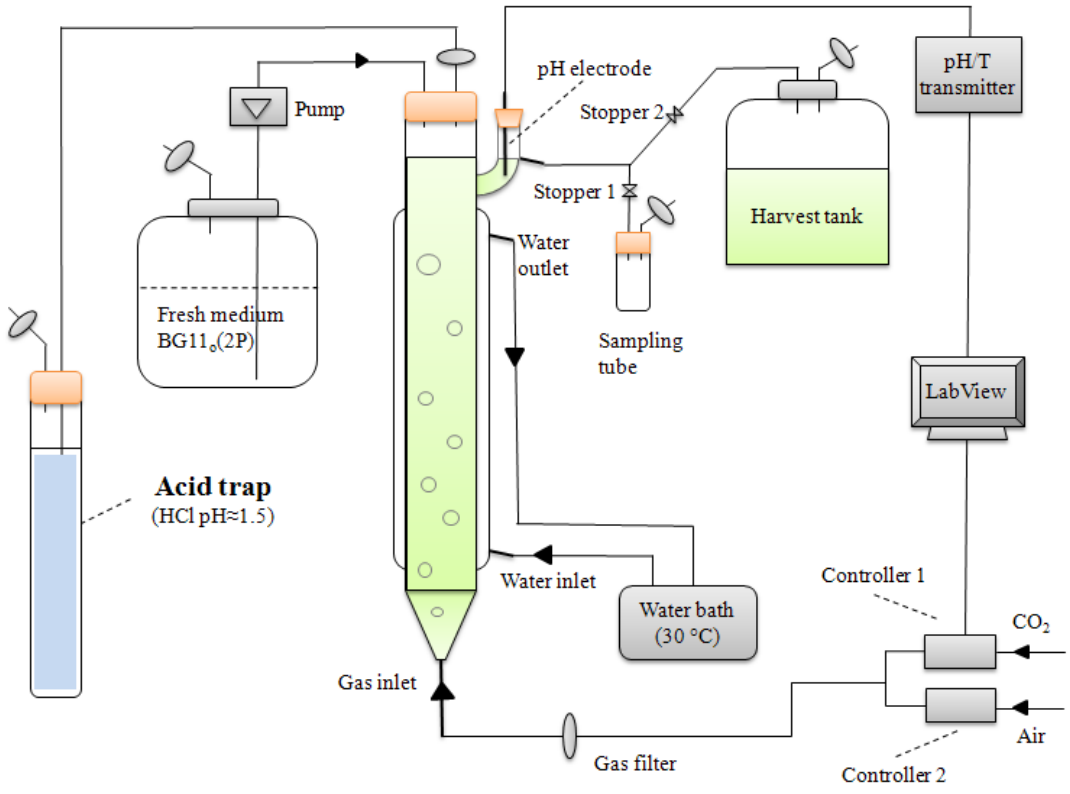


Figure 2−8. Scheme of whole set-up for kinetics of NH_4^+/NH_3 excretion by *A. variabilis* PCC7937-C9 in continuous culture at a dilution rate of 0.3 d^{-1} with an acid trap under pH regulation by adding CO_2.

When taking samples, stopper 1 will be open and stopper 2 closed. At other time, stopper 1 is always closed with stopper 2 open. Gas outlet of the PBR was connected to a 300 mL column with 270 mL HCl solution (pH 1.5) for stripping NH_3 released to gas phase. To avoid NH_3 loss, all the related caps were in silicone and well closed. HCl solution was refreshed each 12 hours for better stripping.

The set-up for kinetics of NH_4^+/NH_3 excretion by *A. variabilis* PCC7937-C9 in continuous culture at a dilution rate of 0.3 d^{-1} with pH regulation by adding CO_2 is shown in **Figure 2–8**. The working volume was 1 L. All the stoppers were in silicone and well-sealed in case of gas loss. Fresh medium $BG11_o$ (2P) were continuously fed to the PBR through a pump at a flowing rate of 12.5 mL min^{-1}, which equals to a dilution rate of 0.3 d^{-1}. Gas came from the bottom at constant air flow rate (210 mL min^{-1}) controlled by Controller 2 (air flow controller) and CO_2 was only injected when pH value detected online is higher than the set pH value depending on Controller 1 (CO_2 flow controller); culture pH was controlled at set pH ± 0.2 pH unit. CO_2 flow rate was controlled to be 10% that of air in order that there was pH decrease but not too much with CO_2 injection. The injected gas was passed through a bacterial gas filter with 0.2 μm pore size (Minisart SRP–25, Sartorius, USA) prior to injection.

The gas outlet was connected to a volume with HCl solution (pH 1.5) for stripping the potential NH_3 released to gas phase from the culture medium. This solution was renewed every 12 hours. Temperature was always controlled at 30±2 °C. 5 mmol L^{-1} axenic MSX was prepared and added either to the fresh medium or directly to the culture in PBR as needed.

2.2.5 Determination of overall volumetric gas transfer coefficients

The volumetric gas transfer coefficient (k_La) can be easily obtained for oxygen, as oxygen sensors are available, with relatively low time response. Then, the k_La for other gas, such as ammonia, could be derived by using their respective mass diffusivities.

The volumetric gas mass transfer coefficients (k_La) for oxygen were determined according to the dynamic method of Taguchi & Humphrey (1966) in the absence of cyanobacteria. The first step of the protocol is to deoxygenate the culture medium by nitrogen

sparging. The second step is to follow the kinetic of "reoxygenation" after re-aeration of the culture. An oxymetric probe (Mettler) is coupled to a transmitter (Mettler) and the data are acquired by the Labview software. The equation of the oxygen transfer from the gas phase to the phase liquid (sterile growing medium) is:

$$\frac{dC_{O_2}}{dt} = k_L a_{O_2}(C'_{O_2} - C_{O_2})$$

Then, after integrating:

$$C_{O_2} = C'_{O_2} - (C'_{O_2} - C_0) \times e^{-k_L a(t-t_0)}$$

and,

$$\ln\left(\frac{C'_{O_2} - C_0}{C'_{O_2} - C_{O_2}}\right) = k_L a\,(t - t_0)$$

The $k_L a$ values are the average of three measurements.

There is a relation between the $k_L a$ for oxygen transfer in the axenic culture medium and the $k_L a$ for ammonia transfer in the same gas liquid system. Indeed in this condition, the specific exchange surface A/V = a is the same, with A is the surface area available for transfer and V is the culture volume;

The contact time t_c between the gas bubbles and the liquid is the same, then, for ammonia, $k_L NH_3 = \sqrt{\frac{D_{NH_3}}{t_c}}$, and for Oxygen, $k_L O_2 = \sqrt{\frac{D_{O_2}}{t_c}}$

the ratio $\frac{k_L NH_3}{k_L O_2} = \sqrt{\frac{D_{NH_3}}{D_{O_2}}}$

where, D_{NH_3} is the diffusion coefficient of ammonia in water,

D_{O_2} is the diffusion coefficient of oxygen in water,

$k_L NH_3$ is the ammonia mass transfer coefficient,

$k_L O_2$ is the oxygen mass transfer coefficient

At 303°K, $D_{NH_3} = 2.20\times10^{-9}$ m^2 s^{-1}, $D_{O_2} = 2.5\times10^{-9}$ m^2 s^{-1}

Under our experimental conditions, $k_L NH_3 = 0.938\times k_L O_2$

The $k_L a$ was determined for oxygen in the mini-photobioreactors.

Table 2−2
k_La values experimentally determined for oxygen and calculated for ammonia (n=3).

Aeration rate (vvm)	k_La oxygen (h^{-1})	k_La ammonia (h^{-1})
0.3	0.20±0.03	0.19±0.02
0.6	0.66±0.08	0.61±0.07
1.2	0.98±0.09	0.92±0.08
2.4	1.36±0.11	1.28±0.10

Then, the relation between the values of the aeration rate and the k_La for ammonia mass transfer is shown in **Figure 2−9**.

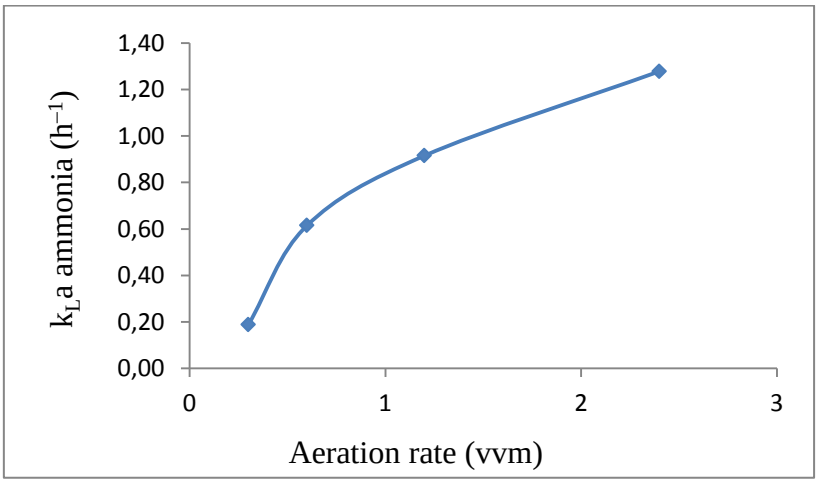


Figure 2−9. Relation between aeration rate and the k_La of ammonia mass transfer.

2.3 Analytical methods and calculations

2.3.1 Biomass concentration and productivity

Biomass concentration X (g L^{-1}) was determined by dry weight (DW) measurement. 5-10 mL samples were filtered over pre−weighed glass fiber−filters (Whatman GF/F) and washed with 0.8 % formate ammonium solution. The filters were dried at 105 °C for 24 h in aluminium trays, cooled down in a desiccator, and the dry weight was measured (Tang et al., 2012).

The cell density was monitored spectrophotometrically at λ=750 nm using spectrophotometer (Perkin Elmer UV/VIS, Spectrometer Lambda 2S, USA). The correlation

between biomass concentration and spectrophotometric absorbance (A_{750}) of both *A. variabilis* strains is shown below.

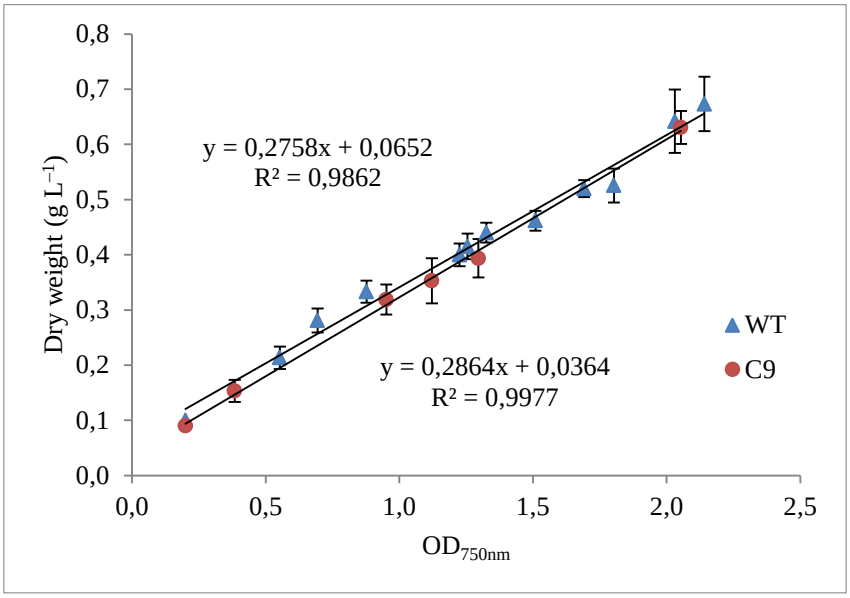


Figure 2−10. Correlation between biomass concentration and spectrophotometric absorbance of *A. variabilis* strains.

In **Figure 2−10**, the correlation between optical density and dry weight of WT and C9 are presented. One optical density unit for WT and C9 equals to about 0.285 g L^{-1}. No apparent difference can be seen.

The biomass productivity Px (g L^{-1} d^{-1}) was calculated as:

$$P_X = D \cdot X \text{ in g } L^{-1} d^{-1}$$

As the S/V ratio is 85 m^{-1} for this photobioreactor, then the illuminated surface productivity in biomass, $P_{X'}$, was calculated as:

$$P_{X'} = \frac{Px}{0.085} \times P_X$$

where, $P_{X'}$ is the biomass productivity, g m^{-2} d^{-1}.

2.3.2 Specific growth rate

In continuous culture, the biomass concentration in PBR, the specific growth rate (μ) of microalga and the dilution rate are related to the equation as follows:

$$\frac{dX}{dt} = \mu X - DX$$

Then, at steady state $\frac{dX}{dt} = 0$, the specific growth rate is equated to dilution rate (Bone, 1971).

$$\mu = D$$

2.3.3 Pigment contents

Samples (2 mL) were centrifuged (13400 rpm, 30 min), and the cells were suspended in 2 mL methanol solution (90% v/v) by vortex (18000 rpm), followed by a dark incubation at room temperature under dim light during 2 h with occasional stirring. The supernatant was clarified by centrifugation (13400 rpm, 10 min) and the optical densities at 470 nm, 665 nm and 750 nm (Spectrophotometer, Perkin Elmer Lambda 2S) were used for determining the chlorophyll *a* (Chl *a*) and carotenoids (Car) concentrations according to the spectrophotometric equations below (Bui et al., 2014).

$$Chla(\mu g.mL^{-1}) = DO_{665nm} \times 13.9$$

$$Car(\mu g.mL^{-1}) = \frac{1000 \times DO_{470nm} - 1.91 \times [Chla]}{225}$$

in which,

$$DO_{665}\,(nm) = DO'_{665} - DO_{750}$$

$$DO_{470}\,(nm) = DO'_{470} - DO_{750}$$

Then, the pigment contents were calculated as below:

$$\text{Chl } a\ (\%) = 100\% \times \frac{10^{-3} \times \text{Chl } a}{X}$$

$$\text{Car}\ (\%) = 100\% \times \frac{10^{-3} \times \text{Car}}{X}$$

where,

Chl *a* (%) is the content of Chl *a*;

Chl *a* is the chlorophyll *a* concentration, μg mL^{-1};

Car (%) is the content of Car;

Car is the carotenoid concentration, μg mL^{-1};

X is the biomass concentration, g L^{-1}.

2.3.4 Specific light supply rate

Specific light supply rate (SLSR, mol photons g X^{-1} d^{-1}) was calculated according to the following relation:

$$SLSR = \frac{\text{PFD}}{\text{X}} \times 0.0864$$

where,

PFD is the photon flux density, in unit μmol photons m^{-2} s^{-1},

X is the biomass concentration, in unit g m^{-2}.

2.3.5 Photosynthetic activities

The kinetic of *in vivo* chlorophyll fluorescence is an indicator of photosynthetic energy conversion in algae and bacteria. PAM Chlorophyll Fluorometers are specialized to measure chlorophyll fluorescence yield with high sensitivity and selectivity. Fv/Fm is the maximal yield of photochemical energy conversion, which shows whether or not cell stress affects PSII in a dark adapted state, and is the most used chlorophyll fluorescence measuring parameter in the world (Baker and Rosenqvist, 2004). In general, greater stress on the cells would result in less availability in open reaction centers and lower Fv/Fm ratio. It i**s** a measurement ratio representing the maximum potential quantum efficiency of PSII complex (Maxwell and Johnson, 2000), which exists only in vegetative cells but not heterocyts. So, it mostly shows us the state of vegetative cells. This test can translate the physiological states of PSII to biophysical parameters under various conditions.

In blue green algae, a P/I curve shows the photosynthetic response of algal communities to varying light intensities. The shape of the curve demonstrates the principle of limiting factors; in low light levels, the rate of photosynthesis is limited by the concentration

of chlorophyll and the efficiency of the light-dependent reactions, but in higher light levels it is limited by the efficiency of RuBisCo and the availability of CO_2 (Smith, 1936).

In vivo chlorophyll fluorescence of photosystem II (PSII) was measured with a Water−PAM (Walz GmbH, Germany). Samples from steady−state cultures were dark adapted for 2 min and then exposed to eight incremental levels of irradiance (49, 75, 109, 165, 245, 346, 479, and 773 μmol photons m^{-2} s^{-1}), each with a 30s light adaptation duration (Cosgrove and Borowitzka, 2006). The P/I curve was fit as Platt's equation (Platt et al., 1980) and values of ETRmax were calculated with WinControl-3 Software (Walz GmbH, Germany).

Electron transport rate (ETR) vs photosynthetic active radiation (PAR) values were fitted as P/I curves according to Platt's equation, i.e.:

$$\mathrm{ETR} = \mathrm{ETR_{mPot}} \cdot \left(1 - e^{-\frac{\alpha \cdot \mathrm{PAR}}{\mathrm{ETR_{mPot}}}}\right) \cdot e^{-\frac{\beta \cdot \mathrm{PAR}}{\mathrm{ETR_{mPot}}}}$$

where, α is initial slope of P/I curve, which is related to quantum efficiency of photosynthesis (electrons/photons), and β is photoinhibition parameter. The values of α, β and ETR_{mPot} are estimated by the fitting procedure.

ETR_{max} is the abbreviation of the Maximum Electron Transport Rate, which gives an expression of PSI, which occurs in both vegetative cells and heterocyts. Relative ETR values are valuable for stress measurements when comparing among different cells, which may be affected mainly by high pH, NH_3 toxicity, scarcity in nutrients, lack or excess in light.

Maximum ETR (ETR_{max}, μmol electrons m^{-2} s^{-1}) is determined by the equation:

$$\mathrm{ETR_{max}} = \mathrm{ETR_{mPot}} \cdot \left(\frac{\alpha}{\alpha + \beta}\right) \cdot \left(\frac{\beta}{\alpha + \beta}\right)^{\frac{\beta}{\alpha}}$$

The "width" was set as 30 s and duration of dark adaptation was set as 2 min according to **Figure 2−11**.

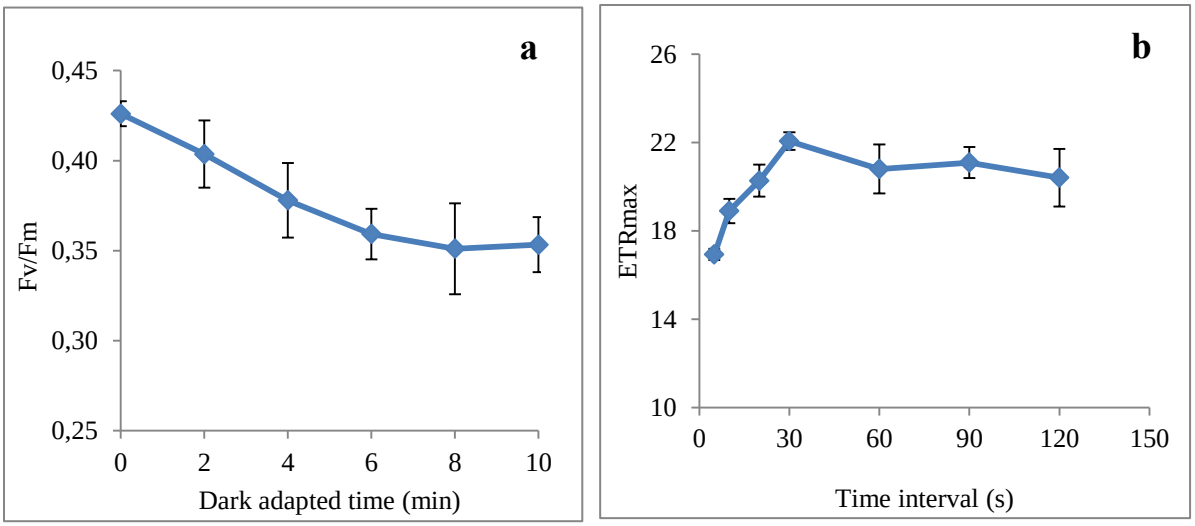


Figure 2−11. The values of (**a**) Fv/Fm as a function of dark adapted time and (**b**) ETR_{max} as a function of duration at each actinic light adaptation of *A. variabilis* PCC7937 cultures.

2.3.6 Dissolved inorganic carbon concentrations and calculation for $CO_{2(aq)}$

The determination of the dissolved inorganic carbon (DIC) was carried out with a TOC Analyzer (Shimadzu, TOC-5000A, Japan). After the centrifugation (13400 rpm, 30 min), the supernatants of samples were injected. Data were calculated by internal software of the TOC Analyzer.

The relative concentrations of all DIC species, dissolved carbon dioxide ($CO_{2(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) in the culture, were calculated from the DIC and the pH values at each steady state with the equations derived from the following reactions according to **Figure 2−12**:

$$[\mathrm{DIC}] = \left[CO_{2(aq)}\right] + [H_2CO_3] + [HCO_3^{-}] + \left[CO_3^{2-}\right];$$

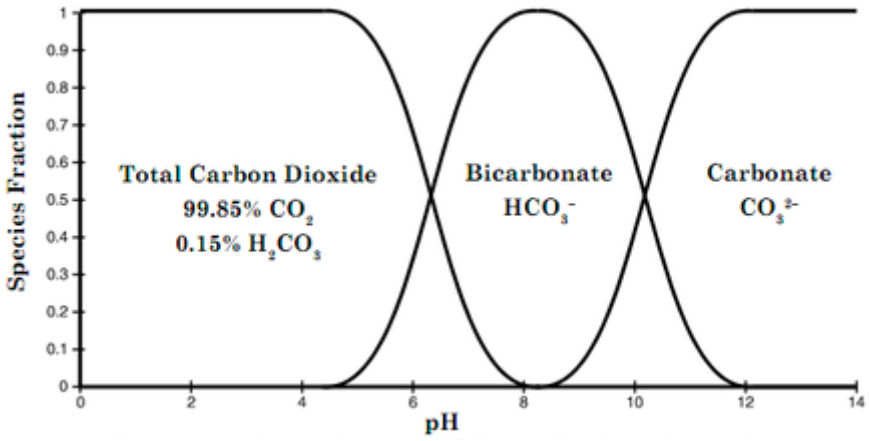


Figure 2−12. Distribution of total carbon dioxide, bicarbonate and carbonate in function of pH.

At constant pressure and temperature, the following equilibriums between $CO_{2(aq)}$, H_2CO_3, HCO_3^- and CO_3^{2-} species apply:

$$CO_{2(aq)} + H_2O \overset{K_0}{\leftrightarrow} H_2CO_3 \overset{K_1}{\leftrightarrow} H^+ + HCO_3^- \overset{K_2}{\leftrightarrow} 2H^+ + CO_3^{2-}$$

$$\text{Where, } K_o = \frac{[H_2CO_3]}{CO_{2(aq)}}, K_1 = \frac{[HCO_3^-]\cdot[H^+]}{[H_2CO_3]}, K_2 = \frac{[CO_3^{2-}]\cdot[H^+]}{[HCO_3^-]}$$

The calculations were done with the constants from Millero and Roy (Millero and Roy, 1997). At equilibrium, $K_0 \approx 1.7 \times 10^{-3}$; then only a small fraction, less than 1.0 % of the dissolved CO_2 is converted to H_2CO_3, with the main fraction as aqueous molecular $CO_{2(aq)}$. K_1 and K_2 were calculated from the following equations (Chen et al., 2016):

$$pK_1 = \frac{6320.813}{T} + 19.568224 \cdot LnT - 126.34048$$

$$pK_2 = \frac{5143.692}{T} + 14.613358 \cdot LnT - 90.18333$$

$$\text{Then, } [CO_{2(aq)}] = [DIC] \cdot \frac{[H^+]^2}{[H^+]^2 + K_1\cdot[H^+] + K_1\cdot K_2}$$

Then, at 30 ℃, pH 6.8, $CO_{2(aq)}$ = 26.59 % DIC, HCO_3^- = 73.35 % DIC.

And, at 30 ℃, pH 8.8, there is no more $CO_{2(aq)}$, and most DIC is in the form of HCO_3^- .

2.3.7 Nutrients concentrations

All nutrients concentrations in culture medium were determined using ionic chromatography system, including a cationic chromatograph (Dionex−1100−IonPac) equipped with a guard column (CG16) and a separation column (CS16), an anionic chromatograph (Dionex−ICS 900−IonPac) equipped with a guard column (AG9−HC) and a separation column (AS9HC) and an external AMMS supplied with sulfuric acid (H_2SO_4, 25 mmol L^{-1}, 1.8 mL min^{-1}). Eluents were a solution of 0.9 mmol L^{-1} Na_2CO_3 and 0.5 mmol L^{-1} $NaHCO_3$ with a flow of 0.9 mL min^{-1} for the anionic chromatography and a solution of 20 mmol L^{-1} H_2SO_4 with a flow of 0.9 mL min^{-1} for the cationic chromatography. Detection was by conductivity and data acquisition and processing were accomplished with the software program Chromeleon.

2.3.8 Determination of ammonium (NH_4^+/NH_3)

The determination of NH_4^+/NH_3, could be done by two methods, either by, ionic chromatography or by the colorimetric method of Solorzano (Solorzano, 1969). They have been compared (**Table2−3**). The colorimetric method was choosen as it could be done by using microplates with a microplate reader. The standard curve was done every week.

Table 2−3
Comparison between two methods of NH_4^+/NH_3 determination.

	Ion chromatography	Solorzano's method
Minimum volume of sample (mL)	4	0.2
Efficiency (min/sample)	50−80	5−15

NH_4^+ was measured according to the indophenol blue complexion calorimetric method of Solorzano (Solorzano, 1969), which has been widely used for ammonium determination (Brouers and Hall, 1986; Kannaiyan et al., 1994; Park et al., 1991; Shi et al., 1987; Thomas et al., 1990; Vincenzini et al., 1986; Zimmerman and Boussiba, 1987). Samples were centrifuged (13400 rpm, 30 min) and the supernatants were immediately analyzed. Solutions with NH_4^+ were diluted for appropriate times according to the concentration of NH_4^+. After 30 min incubation at 40 °C, the absorbance at 630 nm was used to calculate the NH_4^+ concentrations using a standard curve of NH_4Cl.

NH_3 trapped in HCl solution was considered as NH_3 liberated to gas phase according to **Figure 2−13**, so samples of NH_3-gas measured for gaseous NH_3 by the same method. The reaction was done in Eppendorf tubes (1.5 mL), and 150 μL liquid after reaction of each sample was transferred to the 24−well microplate. Absorbance was determined within a Multimode Plate Reader (EnSpire Perkin Elmer 2300, Perkin Elmer Singapore Pte. Ltd., Singapore.) Calibration curve was done with a series of NH_4Cl solutions in precise concentrations of 7.14, 21.4, 35.7, 49.98, 64.26 μmol L^{-1}. Reactants were prepared precisely, kept at 4 °C in a fridge and utilized in less than one month. Reactant one is phenol nitroprusside: dissolve 5.0 g phenol and 3.0 mg sodium nitroprusside in 100 mL distilled water. Reactant two is alkaline hypochlorite: dissolve 2.0 g NaOH in 100 mL distilled water and add 1.08 mL NaClO solution (with 9.6% active chlorine).

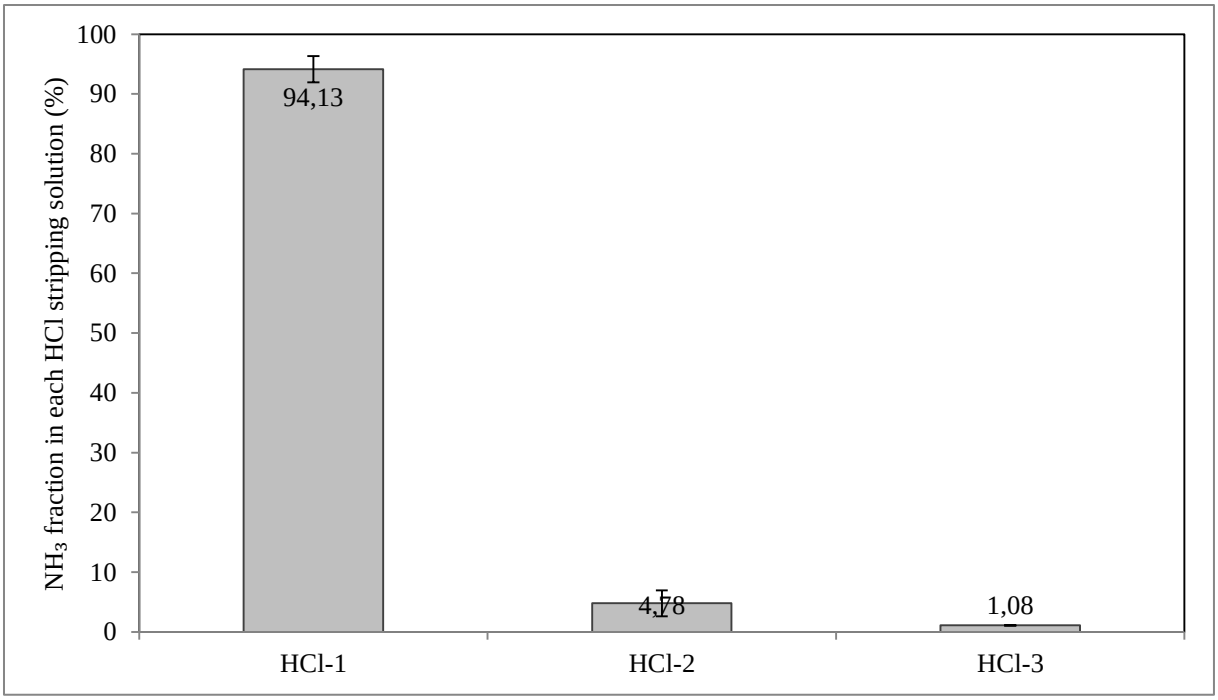


Figure 2−13. NH_3 trapped in three successive HCl solutions (Aeration rate τ=2.0 vvm).

It was shown, by connecting the gas outlet with three successive stripping−tubes with HCl solution (pH 1.5), that about 95 % NH_3 liberated from the cultures could be stripped with only one acid solution at an aeration rate of 2.0 vvm (**Figure 2−13**). If the gas flow rate was lower, there might be more NH_3 stripped in the first HCl solution, and surely the majority would be captured by one HCl solution. Therefore, only one HCl stripping tube was applied to each PBR in the following studies to simplify the manipulation. Additionally, HCl solutions were frequently renewed in case of NH_3 saturation and NH_3 loss.

To be sure both forms of combined nitrogen (NH_3 and NH_4^+) can be determined, all the samples in our study were analyzed immediately after sampling. The basic chemical reactions involved in the Solorzano's method are as below, derived from the Berthelot method (1859). As alkaline hypochlorite is added to the sample all the NH_4^+ would be converted to NH_3, and participate the reactions for coloration.

Sample= mixed of NH_4^+ and NH_3

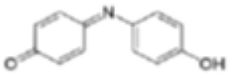
$$OH^- + NH_4^+ \rightarrow NH_3 + H_2O$$

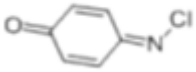
$NH_3 + OCl^- \rightarrow NH_2Cl + OH^-$ = monochloramine formation

$NH_2Cl + C_6H_5OH \rightarrow C_6H_4ONCl$ = N-Chloro-P-Benzoquinoneimine

$C_6H_4ONCl + C_6H_5OH \rightarrow C_{12}H_9O_2N$ = indophenol blue

with, N-Chloro-P-Benzoquinoneimine Indophenol blue





It is important to note that, in this study, "[NH_3+NH_4^+]aq" refers to the sum of both forms of extracellular ammonium ions (NH_4^+) and ammonia molecules (NH_3) maintained in the culture medium as transformation between NH_4^+ and NH_3 exists in solutions (Thurston et al., 1979); "[NH_3]g" refers to the part of NH_3 which is liberated out of the culture and trapped by the HCl solution; "[NH_3+NH_4^+]total" = [NH_3+NH_4^+]aq + [NH_3]g.

It was checked that the addition of MSX has no impact on the determination of ammonium. Indeed the standard curves of ammonium are not modified by the presence of MSX with the standard solutions (Bui, 2013).

Gaseous NH_3 volumetric production (µmol L^{-1}) was calculated as the following equation:

$$T_{NH_{3,t}} = \sum_{\Delta t_0}^{\Delta t} \frac{c_{NH_{3,\Delta t}} \times V_{HCl,\Delta t}}{V_{culture,\Delta t}}$$

Gaseous NH_3 specific production (µmol g X^{-1}) was calculated as the following equation:

$$P_{NH_{3,t}} = \frac{T_{NH_{3,t}}}{X_i}$$

Gaseous NH_3 volumetric production rate (µmol L^{-1} d^{-1}) was calculated as the following equation:

$$r_{NH_{3,t}} = \frac{T_{NH_{3,t+\Delta t}} - T_{NH_{3.,t}}}{\Delta t}$$

Gaseous NH_3 specific production rate (µmol g X^{-1} d^{-1}) was calculated as the following equation:

$$r'_{NH_{3,t}} = \frac{T_{NH_{3,t+\Delta t}} - T_{NH_{3.,t}}}{\Delta t \cdot X}$$

Where, $c_{NH_{3,\Delta t}}$ is the NH_3 concentration stripped in HCl solution during each Δt, $V_{HCl,\Delta t}$ is the volume of HCl solution during each Δt, $V_{culture,\Delta t}$ is the culture volume during each period, $T_{NH_{3,t}}$ is total gaseous NH_3 volumetric production at *t* moment (µmol L^{-1}), X_i is the initial biomass concentration (g L^{-1}).

The excretion rate of NH_4^+ or NH_3 as a unit of μmol mg Chl^{-1} h^{-1} was done as the following equation:

$$r_{NH_{3,t}} = \frac{T_{NH_{3,t+\Delta t}} - T_{NH_{3.,t}}}{\Delta t \cdot X \cdot \%Chl}$$

2.3.9 Morphological observation

Light and fluorescence images were obtained with an Axioskop fluorescence microscope, equipped with digital camera AxioCam MRm and AxioVision Rel software for image processing and Neofluor series objective at 400X magnification (Carl Zeiss, Germany).

2.4 Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Data were treated by Office Excel software to test the homogeneity of variance by Levene Test (package Car). Differences between the groups were statistically analyzed by using one−way analysis of variance (ANOVA) and Tukey's 'Honest Significant Difference' method (TukeyHSD) with 95% family−wise confidence level.

CHAPTER III Diazotrophic biomass production: a comparative study in continuous cultures of *A. variabilis* strains

3.1 Introduction

Heterocytous cyanobacteria are particularly attractive as potential bio−fertilizer and source of chemicals of commercial value, because they are able to do nitrogen fixation and carbon fixation simultaneously. Besides lowering the cost of the culture medium, the ability of fixing dinitrogen restricts the problem of contamination by other microorganisms (Moreno et al., 2003). Additionally, the filamentous nature of these microorganisms facilitates harvesting of biomass. Despite these obvious advantages, available information concerning biomass production of filamentous N_2−fixing cyanobacteria in diazotrophic continuous culture is scarce (Bone, 1971 ; Masuda et al., 2013 ; Moreno et al., 1998 ; Thomas and David, 1971).

As one heterocytous cyanobacterium, the wild type of *A. variabilis* PCC 7937 is a filamentous N_2−fixing freshwater cyanobacterium which, under controlled laboratory conditions, exhibits a high growth rate (about 1.47 d^{-1}), the optimal temperature (30 °C), pH (7.0) and superficial gas velocity (2.0 cm s^{-1}) with the maximum cell concentration (0.79 g L^{-1}) and cell productivity (0.26 g L^{-1} d^{-1}) (Yoon et al., 2008). An ammonium excreting mutant strain of *A. variabilis* PCC 7937 has also been isolated by double random mutagenesis treatments with EMS and characterized (Bui et al., 2014).

In present study, *A. variabilis* PCC 7937 (the wild type, WT) and its mutant C9 were cultivated in airlift photobioreactors with pH regulation in absence of combined nitrogen. A series of growth rates at steady-state in continuous cultures from 0.3 d^{-1} to nearly 3.0 d^{-1} were obtained by setting feeding medium flow rates. The relationships of cell specific growth rate, pigment contents and biomass productivity were analyzed, as well as average irradiance condition, nutrients supply and consumption, and photosynthetic activities. To our knowledge, this is the first time to use continuous cultures system with full environmental conditions control for obtaining data in steady-states at different specific growth rates on *A. variabilis*.

In this study, several steady states were achieved for both strains, but no significant differences between C9 and WT was found except photosynthetic performance. The maximal specific growth rate of both reached about 3.0 d^{-1}. The biomass productivity were, respectively, as high as 0.8 g L^{-1} d^{-1} at D=2.5 d^{-1} for C9 and 0.82 g L^{-1} d^{-1} at D=2.65 d^{-1} for WT, both of which were several times higher than ever studies. From DIC variation, it can be said that the cultures were still not saturated by CO_2, so higher biomass productivity could be obtained if more CO_2 were offered. Moreover, the mutant strain C9 showed better

photosynthetic efficiency comparing to the wild type WT. This study is fundamental for further study in cyanobacteria, no matter for biomass application or development of their metabolites as high biomass productivity is basic.

3.2 Results and discussion

3.2.1 Growth and steady-states

Continuous cultivation of both strains WT and C9 of *A. variabilis* PCC7937 in a 0.7 L PBR was performed with continuous illumination at 110 μmol m^{-2} s^{-1} using white fluorescent light, pH regulated at 6.8 with automatic supply of CO_2, and temperature of 30°C. Continuous cultures were conducted for 120 days for WT and 60 days for C9, because more dilution rates were applied to the test for WT which was carried out in priority to C9. **Figure 3−1** shows the evolution of the biomass concentration at various dilution rates (from 0.32 d^{-1} to 2.85 d^{-1} for WT and 0.5 d^{-1} to 3.0 d^{-1} for C9). Changes in dilution rate of continuous cultures had a significant influence on the steady state optical density and biomass concentration. As expected, elevated dilution rate reduced the steady state optical density and biomass concentration. The value of steady-state optical density and biomass concentration for WT ranged, respectively, from 2.14 to 0.55 and from 0.66 to 0.21 g L^{-1}; while those for C9 were from 2.05 to 0.28 and from 0.63 to 0.03 g L^{-1}, separately. Under present cultivation conditions, it seems that the maximal dilution rate without washing out was at 3.0 d^{-1}, while in former studies the highest dilution rate used is just 0.72 d^{-1}; *A. flos-aquae* have been grown in chemostats for investigating the effect of phosphate-limitation on biomass production at the dilution rates of 0.36 – 0.72 d^{-1}, (Bone, 1971). When grown in BG11o (2P) medium no significant differences appeared in optical density and dry biomass between the mutant strain C9 and the wild type WT n the range of tested dilution rates (**Figure 3−1**).

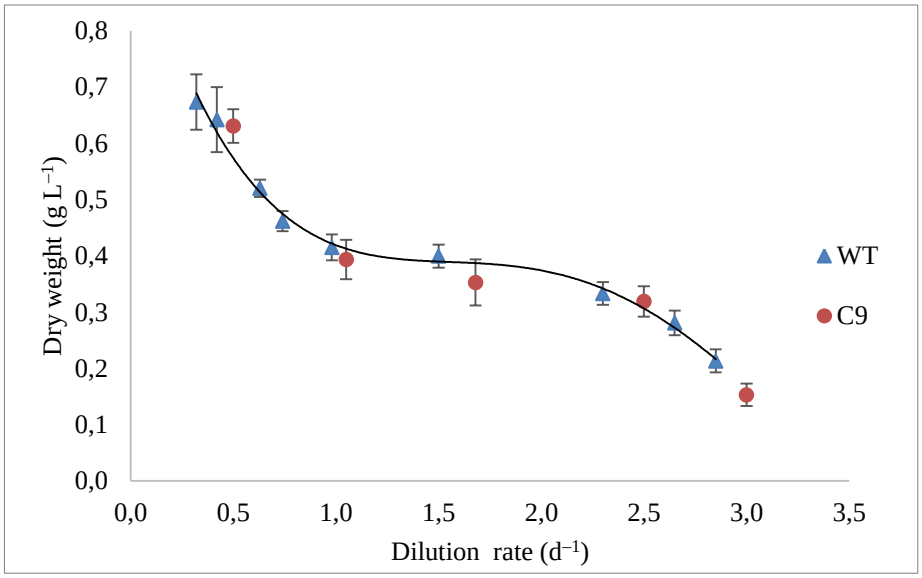


Figure 3−1. Steady state dry weights of both strains WT and C9 of *A. variabilis* PCC7937 at various dilution rates (each data is the mean ± SD of more than three samples. WT: wild type; C9: the mutant strain).

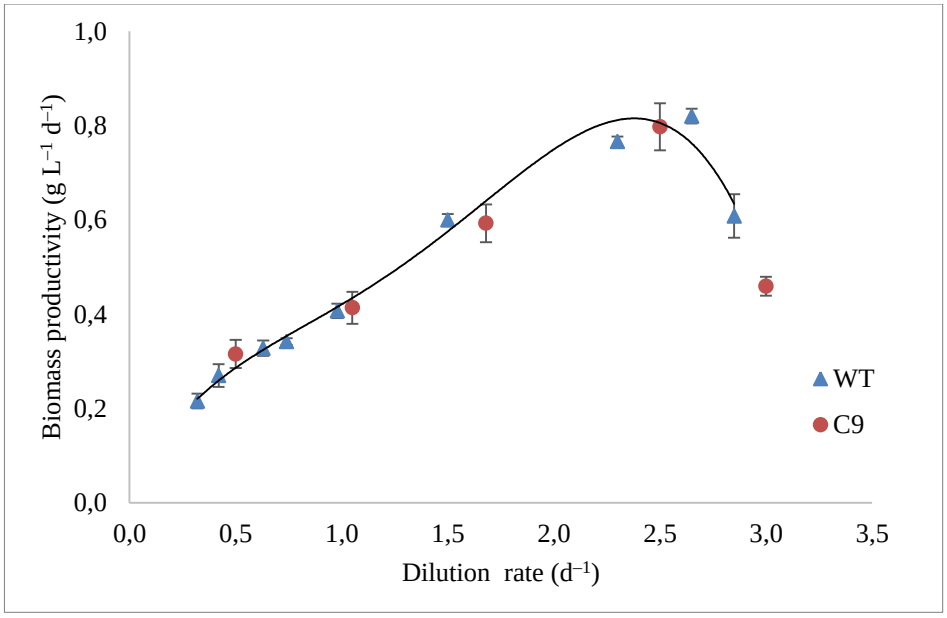


Figure 3−2. Steady state biomass productivities of both strains WT and C9 as a function of dilution rate (each data is the mean ± SD of more than three samples. WT=wild type; C9=the mutant strain).

Figure 3−2 shows the biomass productivity of both strains cultivated under diazotrophic conditions as a function of dilution rate. For the lighting and nutrient supply conditions, the maximal biomass productivity of both WT and C9 was about 0.80 g L^{-1} d^{-1} (9.4 g m^{-2} d^{-1}) at an optimal dilution rate of 2.5 d^{-1}. An earlier study under N_2-fixing conditions showed that the biomass productivity of *A. variabilis* was 0.048 g L^{-1} d^{-1} dry mass in semi-continuous outdoor container with a depth of 25 cm, open to the air and set up on the roof, sparged with air, T=30 °C (Fontes et al., 1985). In batch cultures of *Nostoc* sp.,

maximum biomass of 1.63 g L^{-1} and 2.14 g L^{-1} were respectively obtained in an airlift reactor in $BG11_o$ and in a flat panel reactor in the $BG11_o$, with biomass productivities of 0.54 and 0.39 g L^{-1} d^{-1} (Johnson et al., 2014).

In batch cultures of *Nostoc* sp. with combined nitrogen and carbon, the maximal biomass concentration was only 0.45 g L^{-1} after 10 days at the stationary growth phase (Silva et al., 1994). For *Spirulina* grown in the very nutritious Zarrouk medium in an outdoor minipond (100 m^2 × 0.15 m depth), its productivity was maximal in summer but only 0.15 g L^{-1} d^{-1} during the whole year (Vonshak et al., 1982). The maximal biomass productivity obtained in our study seems to be the highest obtained till now under diazotrophic conditions.

3.2.2 Specific light supply under each steady-state

In continuous cultures, biomass concentrations are lower at higher dilution rates as a consequence of the dilution effect. With the same incident photon flux density (PFD) supplied, the average photons obtained by each cell would be higher. Then, depending on the PFD and biomass concentration, it's clear that the specific light supply rate (SLSR) increased along with increasing dilution rate, as it is shown in **Figure 3−3**. Higher SLSR could be better for the photosynthesis of cells, but there might also be light inhibition if too much. It's easy to see that no difference in SLSR between both strains C9 and WT due to the same PFD supply and similar biomass concentration.

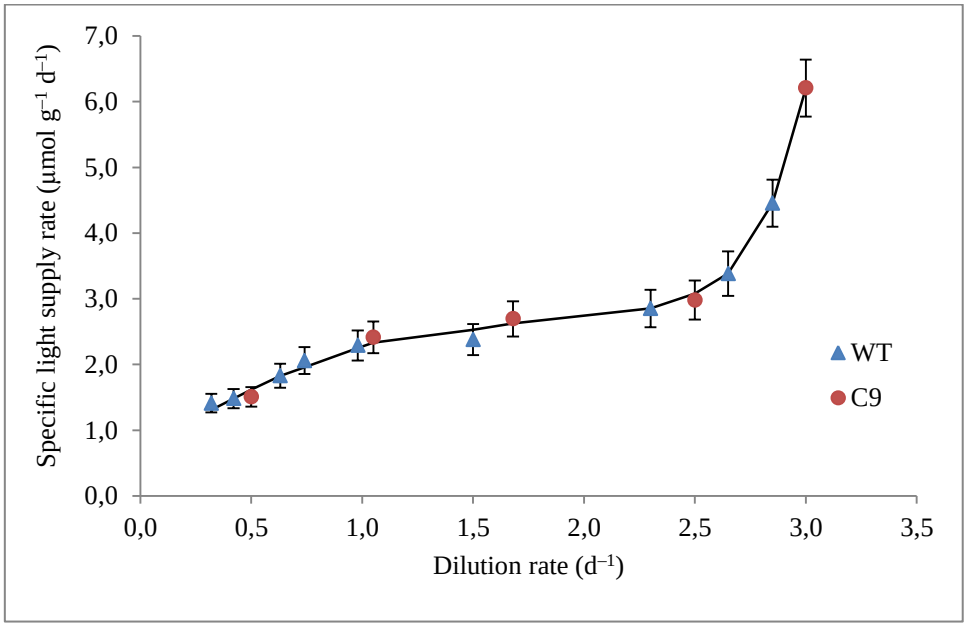


Figure 3−3. Specific light supply rates of diazotrophic *A. variabilis* cultures under successive steady states at constant incident photon flux density.

3.2.3 Photosynthetic performance

As all photosynthetic cyanobacteria, *A. variabilis* PCC7937 has evolved a light-harvesting complex for gathering light from visible part of the solar spectrum, known as photosynthetically active radiation to fuel both PSI and PSII. Their photosynthetic pigments are the chlorophyll a and carotenoids, but also phycobiliproteins. *In vivo* chlorophyll fluorescence analysis reflects the PSII functionality. The ratio of variable to maximal Chl *a* fluorescence (Fv/Fm) of PSII represents the maximal quantum yield of the photosynthetic apparatus (Rastogi et al., 2014). Here, the photosynthetic performances of both strains were also investigated, shown as **Figure 3−4.**

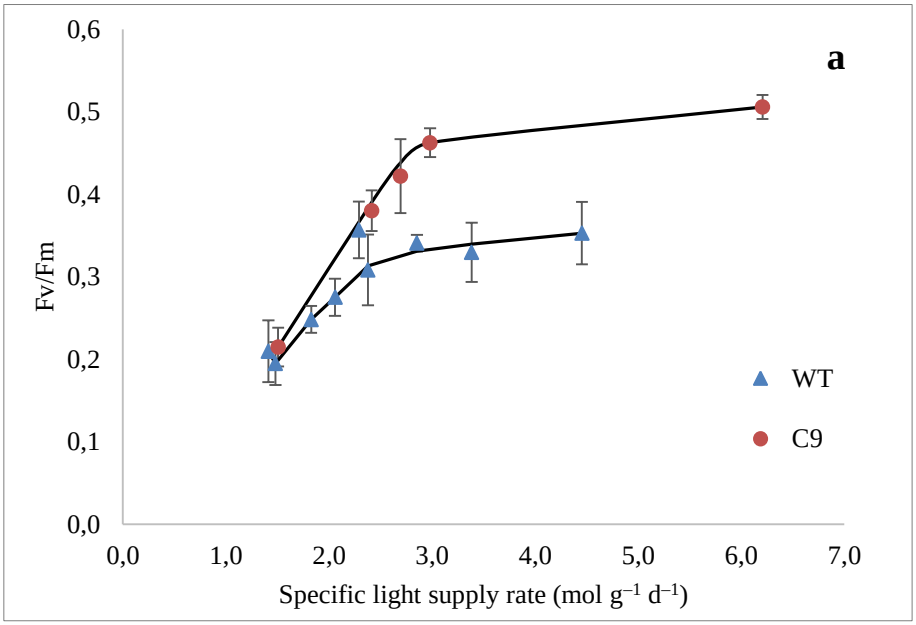


Figure 3−4. Photosynthesis efficiency of both cultures WT and C9 of *A. variabilis* PCC7937 at different specific light supply rates (each data is the mean ± SD of more than three samples. WT=wild type; C9=the mutant strain).

As shown in **Figure 3−4**, the mutant strain C9 has a higher photosynthetic efficiency than the wild type WT seeing from the maximal Chl *a* fluorescence (Fv/Fm). Higher the dilutions rates, higher the differences in Fv/Fm values between both strains.

Basically the evolution of Fv/Fm values reflect the physiological state of the photosynthetic cells. Indeed, Fv/Fm represents the PSII efficiency of cells, which of both strains enhanced with respect of increasing dilution rate and reached a stable state at SLSR levels higher than 3.0. This is predictable because higher dilution rates resulting in lower cell concentration, under which condition the average irradiance availability for every cell were higher as is shown in **Figure 3−3**. However, PSII efficiency of C9 increased much faster than

WT. It's well known that PSII manages light capture by pigment complex, so difference in Fv/Fm implied inconsistency in pigment composition between both strains. For instance, Fv/Fm of C9 was 0.42 at D=2.5 d^{-1}, and that of WT was only 0.35 even at D=2.85 d^{-1}. It is notable that PSII will be diminished by heterocyts in order to protect Nase from oxygen inhibition. Thus, difference in Fv/Fm may also reflect changes in the differentiation of heterocyts from vegetative cells along the filaments of C9 after mutagenesis from WT.

The maximal values of electron transport between PSII and PSI, heterocyt, $ETR_{max,}$ is C9 25.0 at dilution rate around 1.7 d^{-1} for C9 strain (data not shown), but only 19.2 at D=1.3 d^{-1} for the wild-type strain (data not shown). At dilution rates higher than 1.7 d^{-1} (data not shown), ETR_{max} of C9 decreased which could be due to photoinhibition for diluted cultures as is shown in **Figure 3−3.**

3.2.4 Pigment content

It has been shown that the photosynthesis performance of C9 was better than that of WT from previous results. As photosynthesis is dependent on pigment compositions, the Chl *a* and carotene contents were determined. Data are presented in **Figure 3−5**. It is obvious that the Chl *a* and carotene contents of C9 were both higher than those of WT under various SLSR levels. More pigments content are essential for the photosynthesis efficiency.

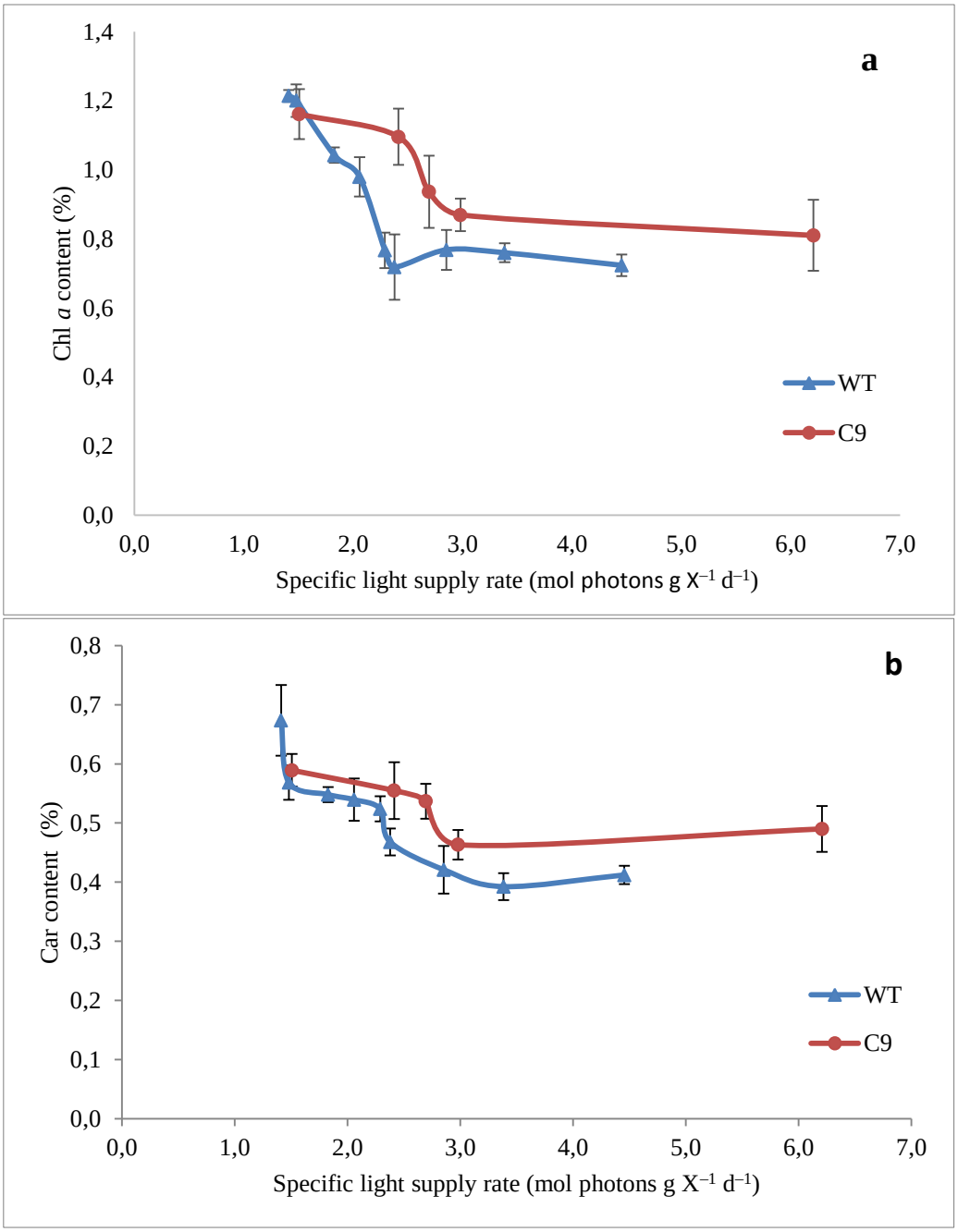


Figure 3−5. Pigment content **(a)** Chlorophyll and **(b)** Carotenoid of both strains under successive steady-states.

Apparent difference in pigment composition between C9 and WT has also been observed visually in batch cultures that C9 was always in blue-green-yellow under diazotrophic autotrophic conditions while WT was blue-green all the time (**Figure 3−6**).

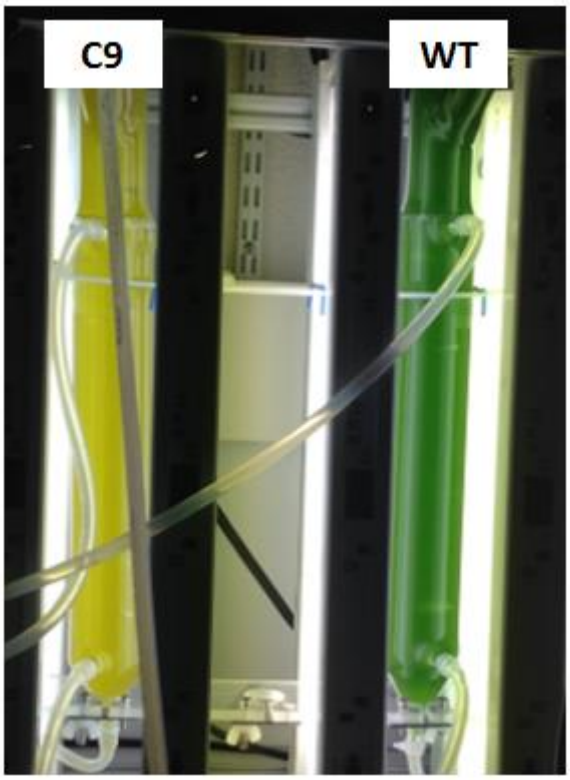


Figure 3−6. Cultures of C9 and WT cultivated in PBRs at 30±2 °C, bubbling with air at 0.3 vvm, with 110 μmol m^{-2} s^{-1} light intensity, in batch culture for 10 days.

These color differences could involve a change in the phycobiliproteins composition of these two strains.

3.2.5 Dissolved inorganic carbon supply

Under photoautotrophic conditions, the growth of cells is relied on the photosynthesis with which inorganic carbon would be fixed. From former part of results, no important difference in biomass production occurred but some difference appeared in the photosynthesis performance and pigment composition. Here, the distribution of dissolved inorganic carbon (DIC) in both cultures under a variety of dilutions was investigated.

In order to supply CO_2 to the cultures frequently, culture pH was controlled at around 6.8, since the pH of $BG11_o$ (2P) medium was around 7.2. To avoid too much decrease in pH, CO_2 regulation time was also controlled.

Table 3−1 shows the distribution of DIC in the cultures of both strains under successive steady states. There was no deviation occurred in DIC concentration and distribution in the tested range of dilution rates. As it is shown, the DIC concentrations were

always lower than 4.0 mg L^{-1}, which might indicates the limitation in carbon supply during the study. Thus, it could be possible to increase the biomass production of *A. variabilis* by elevating DIC supply.

Table 3−1
Distribution of dissolved inorganic carbon in the cultures of both strains under successive steady-states.

WT				C9			
D (d^{-1})	DIC (mg L^{-1})	CO_2 (mg L^{-1})	HCO_3^- (mg L^{-1})	D (d^{-1})	DIC (mg L^{-1})	CO_2 (mg L^{-1})	HCO_3^- (mg L^{-1})
0.53	2.18	0.58	1.60	0.5	1.40	0.37	1.03
0.98	2.85	0.76	2.09	1.05	2.00	0.53	1.47
1.50	2.50	0.66	1.83	1.68	2.72	0.72	2.00
2.65	2.62	0.70	1.92	2.5	3.27	0.87	2.40
2.85	2.85	0.76	2.09	3.0	3.02	0.80	2.22

3.2.6 Nutrients uptake

The residual nutrients concentration were determined in the culture media of WT and the mutant strain C9 at several steady-states (**Table 3−2**). Since the Na^+ concentration in $BG11_o$(2P) medium is lower than the limit of detection by the ionic chromatography, only at the level of around 0.1 mg L^{-1}, no data of Na^+ concentration is shown here.

Table 3−2
Residual nutrients at steady−states in continuous cultures of *A. variabilis* PCC7937-WT and C9 at different dilution rates (mg L^{-1}).

	D (d^{-1})	K^+	Mg^{2+}	Ca^{2+}	Cl^-	P-PO_4^{3-}	S-SO_4^{2-}
$BG11_o$(2P)	--	25.9	9.50	15.6	15.7	33.3	28.5
WT	0.53	22.2±1.6	6.7±0.1	13.4±0.5	16.2±1.3	18.3±1.0	21.8±1.4
	0.98	21.1±1.2	6.8±0.4	13.6±0.9	14.2±1.3	19.5±0.9	23.1±2.3
	1.50	21.5±2.0	7.6±0.5	14.6±1.7	19.4±2.6	22.0±2.0	24.4±2.0
	2.65	22.0±1.6	8.3±0.6	15.3±1.4	15.4±1.1	24.8±1.4	26.8±2.5
	2.85	23.9±0.8	8.9±0.3	16.1±1.3	19.1±2.1	27.7±1.9	28.4±2.2
C9	0.5	21.2±1.3	6.6±0.4	13.1±0.8	16.0±1.0	17.6±1.4	20.8±1.7
	1.05	22.1±1.4	6.9±0.8	14.3±0.7	15.2±1.6	20.3±1.9	23.7±1.3
	1.68	22.8±1.4	7.7±0.7	14.6±1.1	18.4±1.6	23.4±2.2	25.3±2.1
	2.5	22.2±1.9	8.1±0.8	14.9±0.8	17.4±2.1	24.1±2.0	26.5±2.6
	3.0	23.9±2.1	8.8±0.6	16.3±2.3	19.5±1.1	26.7±2.3	28.6±1.2

As expected, at higher dilution rate, more ions are left in the culture medium. It can be seen that all the investigated ions were abundant for cell growth at all the tested dilution rates. The consumption of ions, such as, K^+, Ca^{2+}, Mg^{2+} and Cl^-, seems to be not heavily influenced by cell growth rate, but the consumption of PO_4^{3-} changed clearly on the basis of cell growth rate.

The residual concentrations of all ions in culture medium of the mutant strain C9 were comparable to those of the wild type (WT), and no nutrient was showed to be limiting element for the growth of both strains.

3.2.7 Comparison of biomass productivity with other studies

In **Table 3−3**, our values of biomass productivity of *A. variabilis* cultivated under diazotrophic conditions in continuous cultures are compared with some published data. The productivities of the present study are 66.7% higher than the famous fast-growing eukaryotic microalga *Chlorella vulgaris* which reaches 0.48 g L^{-1} d^{-1} (Sacasa Castellanos, 2013). Another study also showed a range of 0.004 to 0.053 g L^{-1} d^{-1} biomass productivity of several

cyanobacteria (Da Rós et al., 2013), while the biomass productivity attained by *Aphanothece microscopica* fed with combined nitrogen was 0.75 g L^{-1} d^{-1} (Francisco et al., 2010).

Table 3−3
Comparison of biomass productivity among this study and some other studies carried out in laboratory PBRs.

Strain	PBR	Diazotrophic or non	Cultivation model	Px (g L^{-1} d^{-1})	Reference
A. variabilis WT	airlift column	D	C	0.82	this study
A. variabilis mutant C9	airlift column	D	C	0.80	this study
Nostoc sp.	flat panel	D	C	0.27	Johnson *et al.*, 2014
Chlorella vulgaris	tubular PBR	N	C	0.48	Sacasa Castellanos, 2013
Microcystis aeruginosa NPCD-1	flasks	N	B	0.047	Da Rós *et al.*, 2013
Synechococcus sp. PCC7942	flasks	N	B	0.053	Da Rós *et al.*, 2013
Trichormus sp. CENA77	flasks	N	B	0.031	Da Rós *et al.*, 2013
Aphanothece microscopica	bubble column PBR	N	SC	0.75	Francisco *et al.*, 2010
Spirulina maxima LB 2342	airlift PBR	N	B	0.21	Gouveia and Oliveira, 2009

*C=Continuous culture; SC= intermittent regime; B=Batch culture; D= Diazotrophic condition; N=Non-diazotrophic condition; PBR= Photo-bioreactors; Px= biomass productivity.

The biomass productivity of the cyanobacterium *Spirulina maxima* LB 2342 treated with high amounts of reactive nitrogen reached only 0.21 g L^{-1} d^{-1} (Gouveia and Oliveira, 2009). By comparison, it can be indicated that the biomass productivity of *A. variabilis* strains were significantly improved by feeding with CO_2 through pH regulation.

3.3 Conclusion

Cultures of wild type WT and mutant strain C9 of *A. variabilis* were cultivated in continuous scheme at a series of dilution rates with CO_2 supply through pH regulation. Several steady states were achieved for both strains, no significant differences between C9 and WT except photosynthetic performance. The maximal specific growth rate of both reached about 3.0 d^{-1}. The biomass productivity were, respectively, as high as 0.80 g L^{-1} d^{-1} at D=2.5 d^{-1} for C9 and 0.82 g L^{-1} d^{-1} at D=2.65 d^{-1} for WT, both of which were several times higher than ever studies. It can be indicated that CO_2 supply regulated through pH regulation has significantly improved the biomass productivity of both *A. variabilis* strains. From DIC determination, it can be said that the cultures were still not saturated by CO_2, so higher biomass productivity could be obtained if more CO_2 were offered. Moreover, the mutant strain C9 showed better photosynthetic efficiency comparing to the wild type WT. This study is fundamental for further study in cyanobacteria, no matter for biomass application or development of their metabolites as high biomass productivity is basic.

CHAPTER IV Effect of environmental parameters on ammonia production by *A. variabilis* PCC 7937-C9 in batch cultures

4.1 Introduction

During the last decades, a large number of reports have been published concerning N_2 fixation under different conditions, most of which are carried out about the way how heterocyts protect Nase from O_2 attack (Jensen and Cox, 1983 ; Kangatharalingam et al., 1992; Murry et al., 1983; Walsby, 1985), about ammonium production or excretion in presence of MSX (Grizeau et al., 2015; Prasad and Kashyap, 1990; Singh et al., 2008; Thomas et al., 1990), or with immobilized cells (Babu and S. Kannaiyan, 1998; Jeanfils and Loudeche, 1986; Musgrave et al., 1982; Park et al., 1991; Wang et al., 1991), or with cyanobacteria mutants (Brewin et al., 1999; Bui et al., 2014; Thomas et al., 1991, 1990), or about the genes which are related to N_2 fixation and the effects of some environment parameters on ammonium excretion (Cherchi and Gu, 2010; Shukla et al., 1997; Stewart and Rowell, 1975; Yoch and Gotto, 1982) or the activity of Nase determined by acetylene reduction assays (Dilworth et al., 1988; Gallon and A. Falah Hamadi, 1984; Hardy et al., 1973; Stewart and Rowell, 1975). Progress has been made, but ammonium productivities are still low and not cost-effective for industry production.

Glutamine synthase (GS, from sheep brain) is markedly and irreversibly inhibited by methionine sulfoximine (MSX), a structural analog of methionine. In contrast to methionine sulfone, MSX inhibits the enzyme. The rate at which irreversible inhibition is established can be decreased by exogenous supply of glutamate. It is unaffected by the presence of NH_3 (or hydroxylamine), but a mixture of both glutamate and NH_3 can effectively prevent the inhibition. Mishra (2003) has also illustrated that glutamine but not NH_3 is directly involved in the regulation of heterocyt differentiation, interheterocyt spacing pattern and N_2 fixation in *Anabaena*. The inhibition of GS by MSX requires the presence of ATP (adenosine triphosphate) and Mg^{2+} or Mn^{2+} and is associated with the cleavage of ATP to adenosine adenosine diphosphate (ADP). The inhibited enzyme contains close to 8 moles each of a phosphorylated derivative of MSX and ATP, which are tightly bound to the enzyme but which can be released by brief heating at 100 °C or by the treatment of perchloric acid. The phosphorylated derivative of MSX can also be converted by the treatment of acid or several phosphatases to equimolar quantities of inorganic phosphate and MSX (Ronzio et al., 1969). These findings indicate that the irreversible inhibition of GS by MSX is associated with its phosphorylation and the tight binding to the enzyme of MSX phosphate and ADP.

The addition of exogenous MSX to N_2−fixing cultures of the cyanobacteria *A. cylindrica* results in over half of the newly fixed NH_3 being released into the medium (Zimmerman and Boussiba, 1987). MSX inhibits GS activity, but it has negligible effect on alanine dehydrogenase and glutamate dehydrogenase activities under N_2−fixing conditions. In the presence of MSX, intracellular pools of glutamate and glutamine decrease, those of aspartate and alanine + glycine show little change, when the NH_3 pool increases. MSX alleviates the inhibitory effect of exogenous NH_4^+ on Nase synthesis and heterocyt production. The results suggest that in N_2-fixing cultures of *A. cylindrica* the primary NH_3 assimilating pathway involves GS, and probably glutamate synthase (GOGAT), and that the repressor of Nase synthesis and heterocyt production is not NH_4^+ but is GS, GOGAT, or a product of their reactions (Stewart and Rowell, 1975).

However, as shown in **Chap I**, very few literatures can be found concerning the effect on the pH of the culture and, eventually, the NH_4^+/NH_3 distribution after its excretion. It has been reported that no pH variation occurred when *A. variabilis* was cultivated under N_2-fixing condition, but pH increased when fed with combined nitrogen (Bui et al., 2014). The mutant strain *A. variabilis* PCC7937-C9 was used for this study, because it was shown to produce NH_3 in the presence of MSX for long term (Bui et al., 2014).

The process of NH_4^+/NH_3 release was studied as we are expecting that this NH_3 release could be closely pH−related (**Hypothesis 1, page 75**). T−related, light−dependent and also aeration-dependent, as the effects of environmental parameters on such a process are still relatively unknown. The study has taken in account parameters including temperature, MSX concentration, PFD-Photo flux density and gas flow rate. Indeed, we were expecting a production of combined nitrogen, both in the culture medium and in the gas phase (**Hypothesis 2, page 77**). Their impacts on the photosynthesis efficiency were also considered.

4.2 Results and discussion

From previous results, the pH variations within C9/WT cultures cultivated in $BG11_o$ medium were less than ± 0.2 UpH, even cultivated for more than 2 weeks in batch cultures. However, significant pH variations were observed when cells were cultivated in presence of MSX, as is shown in **Table 4−1**. In order to check if the pH variation was induced by MSX addition, another test was also done by directly adding MSX to $BG11_o$ medium, no pH variation was observed, either.

Moreover, the wild type strain WT of *A. variabilis* was shown to be sensitive to MSX that it would die after several days of cultivation in presence of MSX, while the mutant strain C9 showed good resistance to MSX (Bui et al., 2014).

Table 4−1
Relation between pH variation and cultivation condition

Medium	ΔpH (unit d^{-1})
$BG11_o$ + MSX	± 0.2
$BG11_o$ + WT	± 0.2
$BG11_o$ + C9	± 0.2
$BG11_o$ + WT + MSX	+ 2.6 (increase)
$BG11_o$ + C9 + MSX	+ 2.7 (increase)

* Biomass concentration 0.2 − 0.5 g L^{-1}

Since addition of MSX to *A. variabilis* cultures induced pH increase, hypothesis of gaseous NH_3 production related to pH was confirmed in batch culture by pulse regulation of pH.

In the next part of the work, pH increase was observed after MSX addition to diazotrophic cultures of *A. variabilis*. NH_3 was detected in gas phase and stripped by HCl solution.

4.2.1 Basis for gaseous NH_3 production related to pH variations

When cultivated under diazotrophic condition, the pH of the culture of *A. variabilis* PCC7937-C9 is generally stable around 6.8. In contrast, pH increase was observed after the addition of MSX in our pre-experiment, shown in **Figure 4−1a**. This figure indicates that MSX addition induced pH variation in the culture. As reported by Bui et al. (2014), extracellular NH_4^+ was present in the culture medium when treated by MSX, then we supposed that there should be relation between NH_3 production and the pH variation. This surmise can be theoretically explained by the inter-conversion between NH_3 and NH_4^+ when dissolved in water, shown as **Eq. 1−4**, as NH_3 was proved to be the first product of N_2 fixation (Stewart and Rowell, 1975).

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \qquad \textbf{Eq. 1−4 (page 76)}$$

$$pK_a = \frac{0.09108+2729.92}{(273.2+T)} \qquad \textbf{Eq. 1−5 (page 76)}$$

$$pK_a = pH + \log\frac{[NH_4^+]}{[NH_3]} \qquad \textbf{Eq. 1−6 (page 76)}$$

The increase of pH could also lead to the equilibrium change of the distribution to which NH_3 forms NH_4^+, since it depends on the pH of the solution, see **Figure 4−1b**.

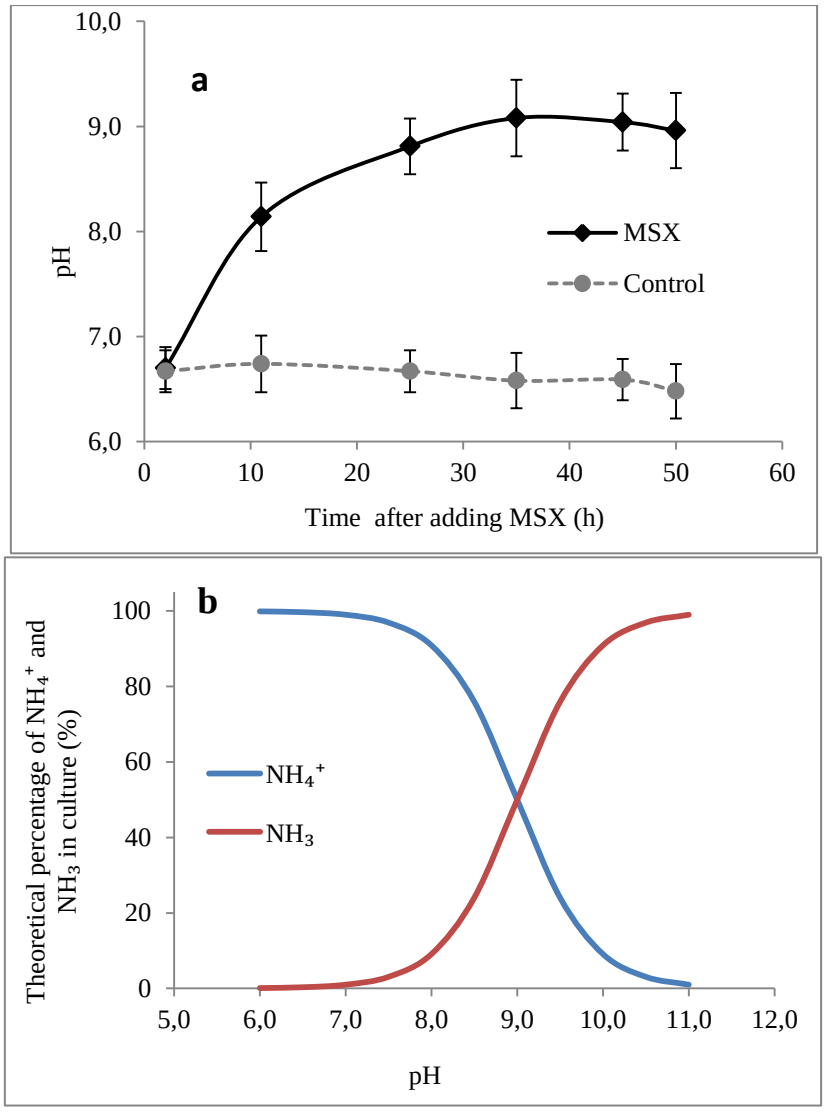


Figure 4−1. (a) Variation of pH of *A. variabilis* cultures in presence of MSX in batch culture and **(b)** distribution of $[NH_4^+]$aq and $[NH_3]$aq as percentages of the $[NH_4^+/NH_3]$ aq as a function of pH (MSX was added to the test at a final concentration of 10 µmol L^{-1} at 2h).

According to **Eq. 1−5** (Körner et al., 2001), the acid dissociation constant pKa is 9.0 at 30 °C. Then, the percentage of NH_3/NH_4^+ in the culture as a function of pH was calculated

according to **Eq. 1−4** and **Eq. 1−6** (Körner et al., 2001). Then, at high pH condition induced by MSX, there should be more $[NH_3+NH_4^+]aq$ in the form of $[NH_3]aq$.

Therefore, the hypothesis established in **Chap I** about the mechanism of gaseous NH_3 production from N_2 in *A. variabilis* culture, was illustrated in **Figure 1−9 (page 78)**. NH_3 is produced after N_2-fixation, and excreted out of cells in the form of NH_3 molecule when GS is inhibited by MSX (Böhme, 1998 ; Sah, 2008). The pH increase seems to indicate that there is a release of NH_3 in the medium, which, according to the actual pH value, is partially transformed into NH_4^+. Then, some of the undissociated NH_3 might be released to the gas phase, especially the culture bubbling with air.

If there is a release of NH_3 ($[NH_3]g$) in the gas phase-outlet, it could be interesting for a potential application of fixed-nitrogen as bio-fertilizer. To make clear this speculation, the experimental setup was built with HCl solution for trapping potential gaseous NH_3 ($[NH_3]g$) from the gas-outlet.

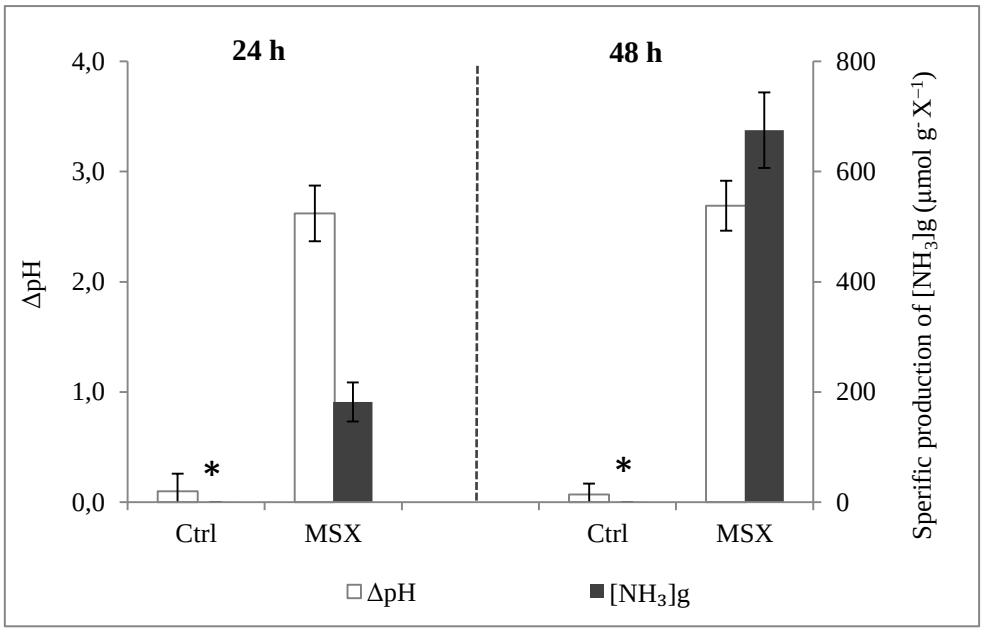


Figure 4−2. Gaseous NH_3 production and ΔpH in batch cultures of the mutant C9 in absence/presence of MSX (Ctrl = without MSX; *= no gaseous NH_3 detected; $[NH_3]g$ = NH_3 liberated to the gas phase and stripped by HCl solution).

Since MSX is toxic, then it would have to be separated from the culture prior to use of NH_3/NH_4^+ as a bio–fertilizer. The observation of gaseous NH_3 production in present study would be meaningful, since the gaseous NH_3 could be used directly without the necessity of MSX separation process.

Note that during the whole process, no gaseous NH_3 was detected in the control cultures (without MSX), the pH increase of 2.6 in the test cultures were not observed in the absence of MSX (**Figure 4−2)**. This observation is in accord with our hypothesis that there could be pH variation along with ammonia excretion after MSX addition. To furtherly understand what happened during the process, kinetics of pH, $[NH_3+NH_4^+]aq$ and $[NH_3]g$ in free cell cultures of *A. variabilis* C9 were investigated.

4.2.2 Kinetics of gaseous NH_3 production and pH pulse regulation in batch cultures

4.2.2.1 First observation of gaseous NH_3 production with pH increase

Figure 4−3 shows gaseous NH_3 was indeed released to gas phase from *A. variabilis* batch culture and can be stripped by the HCl solution. Gaseous NH_3 could be detected after 24 hours bubbling with air into the batch culture. During two days cultivation, gaseous NH_3 production reached more than 600 µmol g X^{-1}. Such data have never been reported in cyanobacteria cultures. The MSX treatment was used for the demonstration of the proof of the concept; although technically feasible it has hindered for long time industrial applications.

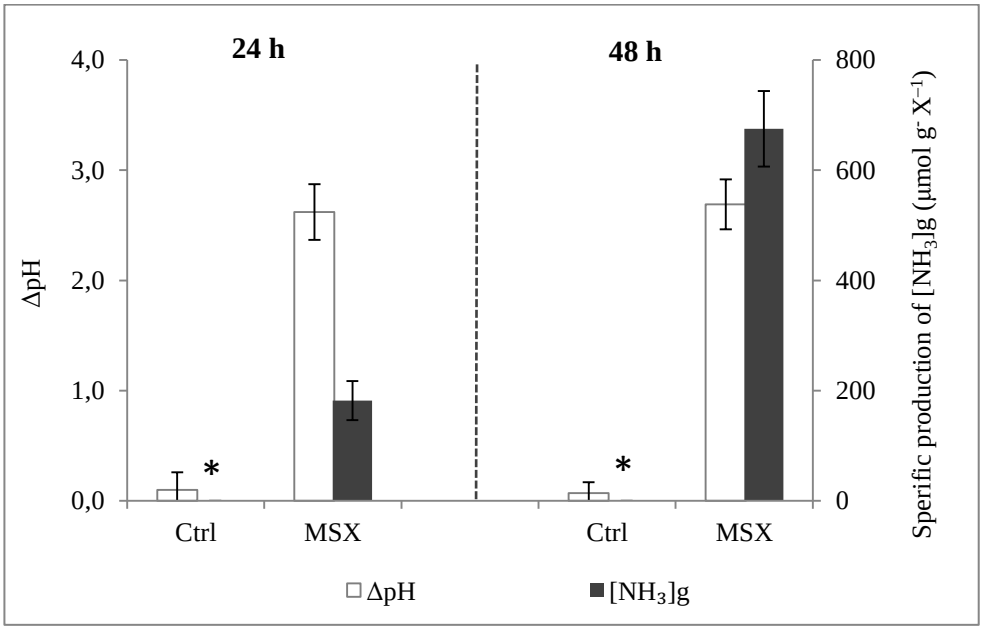


Figure 4−3. Gaseous NH_3 production and ΔpH in batch cultures of the mutant C9 in absence/presence of MSX (Ctrl = without MSX; *= no gaseous NH_3 detected; $[NH_3]g$ = NH_3 liberated to the gas phase and stripped by HCl solution).

4.2.2.2 Kinetics of gaseous NH_3 ($[NH_3]g$) production without pH control

A kinetic study was carried out for 4 days in free cell cultures of *A. variabilis* to address the relation between pH and the production of$[NH_3]g$ and $[NH_4^+/NH_3]$ aq (**Figure 4−4)**.

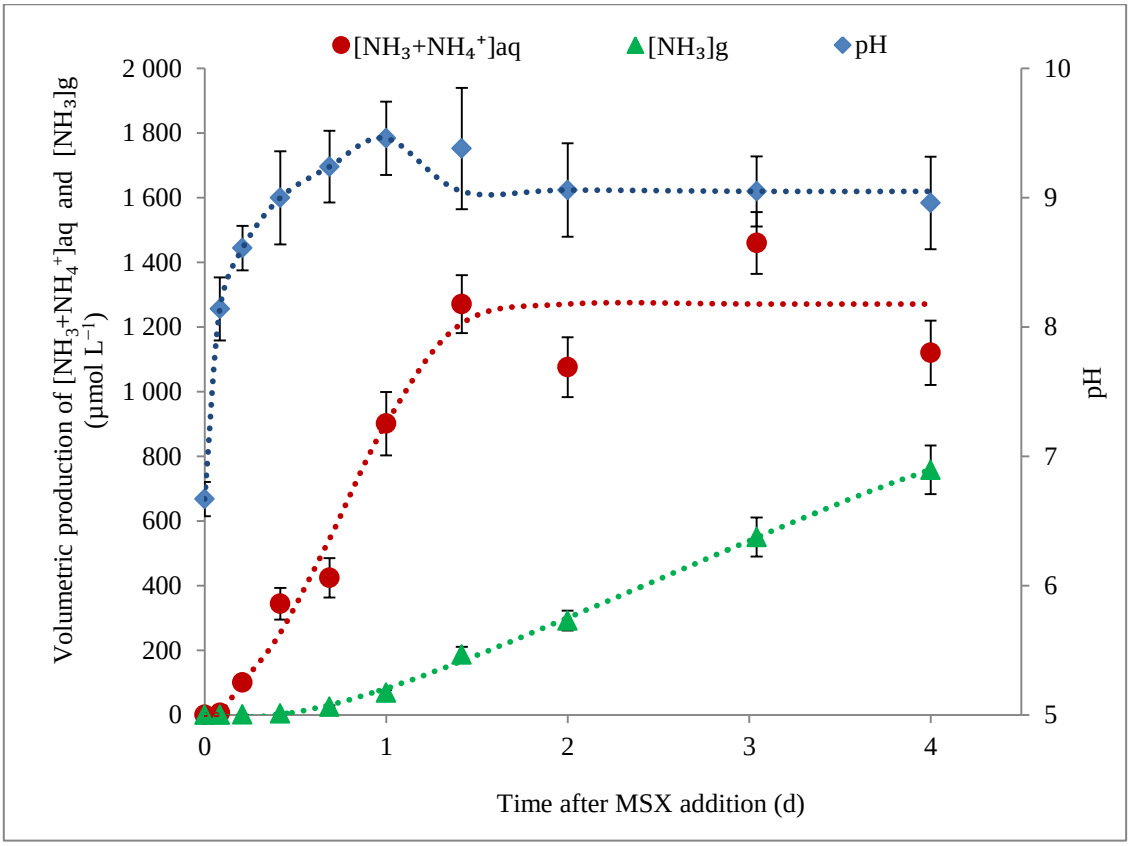


Figure 4−4 a. Kinetics of $[NH_3]g$ production, $[NH_4^+/NH_3]$ aq production and pH variation of the mutant C9 batch cultures in presence of 10 µmol L^{-1} MSX (T=30 °C, PFD =100 µmol m^{-2} s^{-1}, τ=0.3 vvm; MSX was added to the culture at 0 day).

According to the pH variation of cultures with addition of MSX under diazotrophic condition, the whole process comprises two stages, where the culture pH rose up sharply from 6.8 to 9.4 in 1 day after the addition of MSX, followed by a stable level and maintained at around 9.0 for nearly 3 days.

From **Figure 4−4a**, the variation of pH seems to be related to the production of $[NH_3+NH_4^+]$aq. When the $[NH_4^+/NH_3]$aq increases, the pH increases, and vice versa. The increase of pH could be due to NH_3 excreted from the cell to culture medium and dissolved in, as explained by our hypothesis**.** The volumetric production of $[NH_4^+/NH_3]$aq reached 1460 µmol L^{-1}, while that of $[NH_3]g$ was up to 758 µmol L^{-1}. By comparing $[NH_3]g$ production with the culture pH and $[NH_4^+/NH_3]$aq production, it could be found that the major part (91%)

of gaseous NH_3 was produced at high pH around 9.0 from day 1 to day 4, when high amounts of $[NH_4^+/NH_3]aq$ was produced.

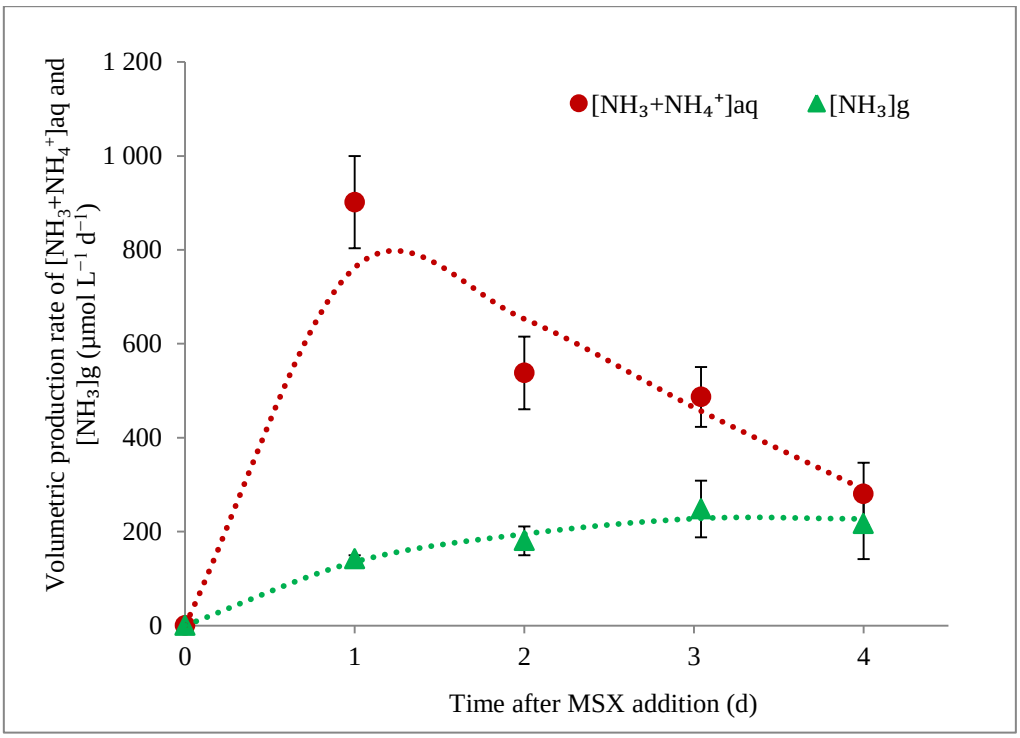


Figure 4−4 b. Volumetric production rate of $[NH_4^+/NH_3]aq$ and $[NH_3]g$ in batch cultures of the mutant C9 in the presence of 10 µmol L^{-1} MSX (T=30 °C, PFD =100 µmol m^{-2} s^{-1}, τ= 0.3 vvm; MSX was added to the culture at 0 day).

Figure 4−4b shows the kinetics of volumetric production rates of $[NH_3+NH_4^+]aq$ and $[NH_3]g$. The maximal production rate of $[NH_4^+/NH_3]aq$ appeared 24h after MSX addition, with a maximal value of reached 902 µmol L^{-1} d^{-1}. Then it decreased continuously during the next 3 days. By contrast, $[NH_3]g$ production rate kept increasing during the first 3 days and reached a stable level of 227 µmol L^{-1} d^{-1}. These curves seem to confirm our hypothesis that fixed nitrogen is excreted out of cells and released to the gas phase directly in the form of NH_3, mainly when the pH reached high levels. Thus, the gaseous NH_3 production could be also an interpretation of the decrease of $[NH_4^+/NH_3]aq$ production rate. It is worth to note that very little gaseous NH_3 was produced at the early stage when both pH and $[NH_4^+/NH_3]aq$ were increasing. Then, the production of gaseous NH_3 could be limited to the pH or $[NH_4^+/NH_3]aq$ production, or both.

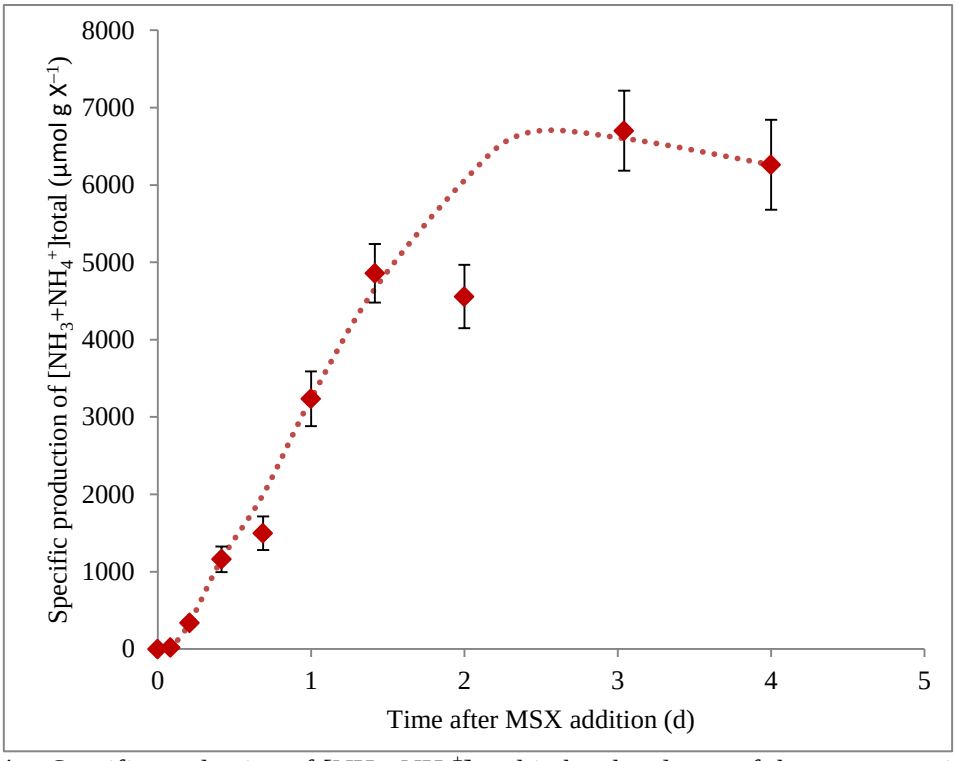


Figure 4−4 c. Specific production of $[NH_3+NH_4^+]$total in batch cultures of the mutant strain C9 in the presence of 10 µmol L^{-1} MSX; $[NH_4^+/NH_3]$ total = $[NH_4^+/NH_3]$ aq +$[NH_3]$g (T=30 °C, PFD =100 µmol m^{-2} s^{-1}, τ= 0.3 vvm; MSX was added to the culture at 0 day).

Since biomass is the base for nitrogen fixation and ammonia production, specific production of total amount of NH_3 and NH_4^+ (namely $[NH_4^+/NH_3]$total) were also analyzed in **Figure 4−4c**. It is clear that the maximal specific production of $[NH_4^+/NH_3]$ total, including the combined nitrogen released in water and gas phases, appear at 48h, with a value of about 6.6 mmoles g X^{-1}. This level was stable during the next two days. From the tendency of $[NH_4^+/NH_3]$ total specific production rate in **Figure 4−4c**, optimal specific production rate seems to occur after an incubation time of 24h. Therefore, the specific production of 24h and 48h would be determined in the next assays, for studying the effects of environmental parameters on the production of $[NH_4^+/NH_3]$aq and $[NH_3]$g.

4.2.2.3 Pulse regulation of pH for $[NH_3]$g production

From our previous data, $[NH_3]$g production rate seems higher at high pH level. So, if pH could increase to a high level earlier, $[NH_3]$g might be detected earlier. In order to confirm that hypothesis, two cultures were started from the same inoculum of *A. variabilis* PCC 7937-C9, one as control and the other where the pH was artificially increased by supplying NaOH after two days of incubation. The productions of extracellular gaseous ammonia were analyzed.

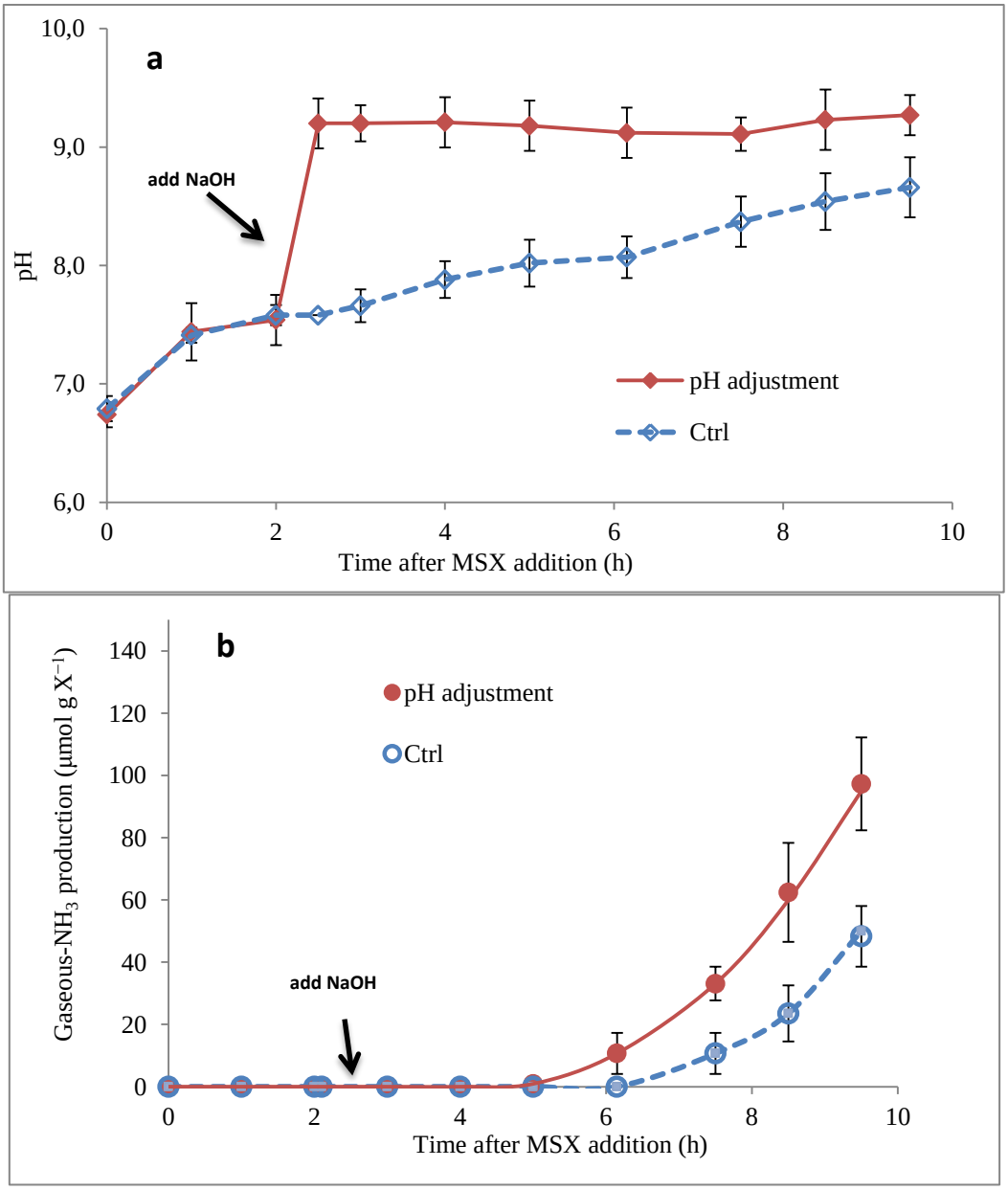


Figure 4−5. (a) pH variation and **(b)** $[NH_3]g$ production in pH pulse regulated cultures of the mutant C9 comparing to the control (T=30 °C, PFD=100 µmol m^{-2} s^{-1}, τ=0.3 vvm; MSX was added to all the assays at 0 h with a final concentration of 10 µmol L^{-1}; 0.2 mol L^{-1} NaOH solution was added to the pH pulse regulation assays at 2.5 h).

Figure 4−5a shows the pH variation of the culture with or without pH adjustment during 10 hours. The pH of the culture was adjusted to about 9.2 by NaOH addition, whereas the control was maintained at pH 7.6. In the following 7 hours, the pH of the test assay kept at the level 9.2, while that of the control increased slowly from 7.6 to 8.66.

In the meantime, $[NH_3]g$ production was measured (**Figure 4−5b)**., The gaseous NH_3 was detected 1.3 h earlier in the test assay than in the control culture, thanks to the addition of NaOH. This confirmed our hypothesis that the gaseous NH_3 could be produced in advance by

increase of pH in *A. variabilis* cultures. Moreover, this proved the possibility of promoting gaseous NH_3 production by regulating culture pH.

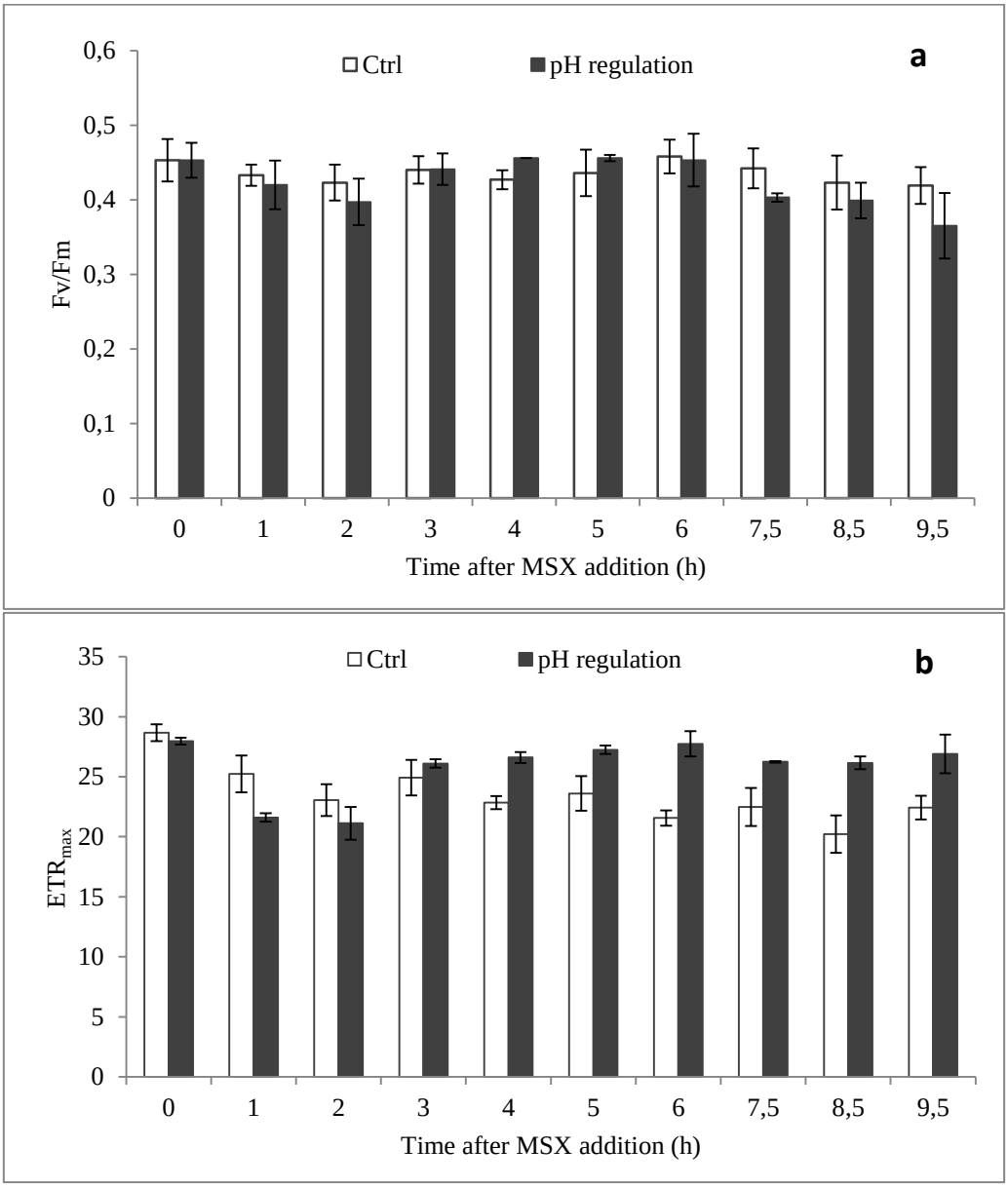


Figure 4−6. Photosynthesis characteristics **(a)** Fv/Fm and **(b)** ETR_{max} of the cells with/without pH regulation after MSX addition in batch cultures with local $BG11_o$ medium in short term (T =30 °C, PFD=100 μmol m^{-2} s^{-1}, τ=0.3 vvm, cultures from chemostat, MSX was added to each assay at 0 h with a final concentration of 10 μmol L^{-1}; 0.02 mol L^{-1} NaOH solution was added to the pH regulation assays at 2.5 h).

The photosynthesis efficiency values of the cell cultures used for the assay with the pH adjustment are presented in **Figure 4−6**. For PSII efficiency as measured by Fv/Fm we observed no obvious difference between the test and the control during the first 6 hours. Then the cultures in the test group showed lower PSII efficiency than those of the control. In terms

of ETR_{max} in **Figure 4−6(b)**, the maximal electron transport of the test culture had higher rates than those of the control culture. This seems to indicate that NaOH addition could enhance electron transport rate but reduce PSII efficiency.

These results show that the pulse pH adjustment in the batch cultures could promote the production of gaseous NH_3.

Then, a better technique could be used in PBR system to continuously control the pH at a set point for further understanding the pH effect on gaseous NH_3 production and cell viability.

4.2.3 Effect of environmental parameters on NH_4^+/NH_3 excretion in short term

4.2.3.1 Effect of MSX concentration

Ammonia excretion was generally induced by a treatment with 10 − 50 μmol L^{-1} MSX on wild–type or mutant strains of heterocytous cyanobacteria (Grizeau et al., 2015). From our pre–experiments, it is confirmed that the extracellular ammonium concentrations were under the detection limit of the analytical method in N_2 growing cultures. To induce excretion of fixed nitrogen, MSX is widely applied to N_2–fixing cyanobacteria (Mishra, 2003 ; Singh et al., 1983 ; Stewart and Rowell, 1975 ; Thomas et al., 1990). For various strains of *Anabaena*, MSX was applied in concentrations ranging from 1 to 55 μmol L^{-1} (Mishra, 2003 ; Stewart and Rowell, 1975), among which some concentrations of MSX could completely inhibit the GS activity. Therefore, it is important to find out the suitable MSX concentration for the excretion of NH_4^+/NH_3 under the condition of our study. The effect of MSX concentration on ammonium excreting ability was checked in terms of specific production of $[NH_4^+/NH_3]$ aq and $[NH_3]$g. Photosynthesis efficiency was also determined at the same time for better evaluating the growth of the cultures.

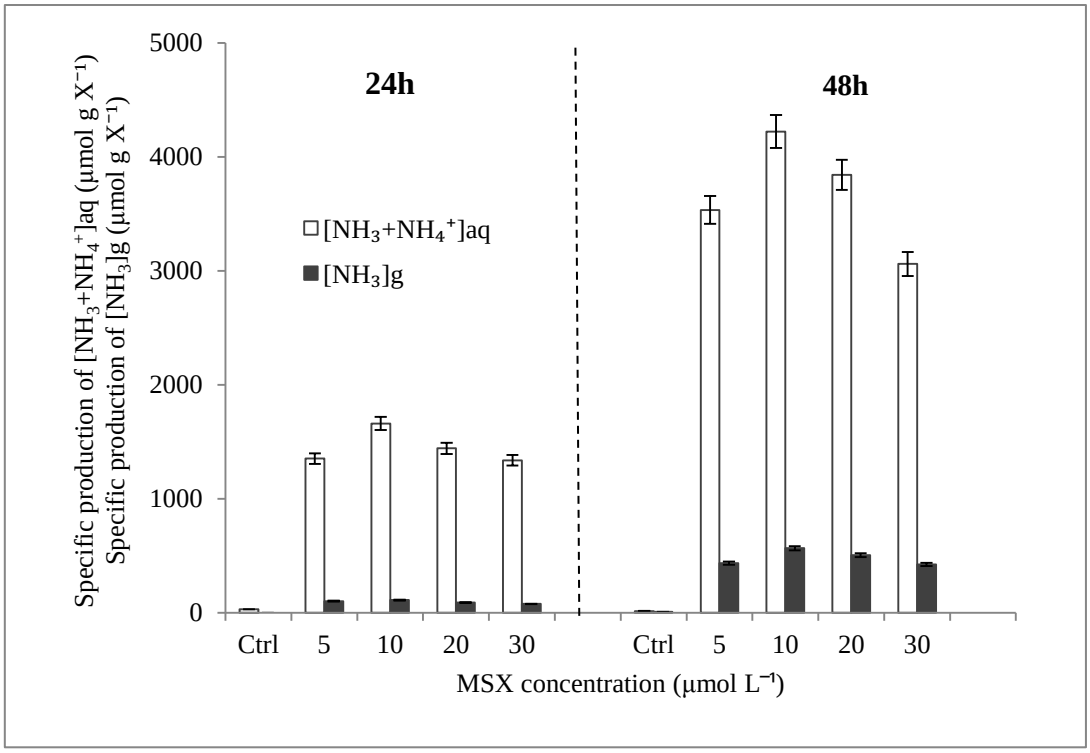


Figure 4−7 a. Specific production of $[NH_4^+/NH_3]$aq and $[NH_3]$g by the mutant C9 in presence of different concentrations of MSX, Ctrl= no MSX addition (T= 30 °C, PFD= 50 µmol m^{-2} s^{-1}, τ=0.3 vvm).

The production of $[NH_3+NH_4^+]$aq and $[NH_3]$g 24h/48h after addition of different concentrations (0, 5, 10, 20, 30 µmol L^{-1}) of MSX is shown in **Figure 4−7a**. In this assay, cultures in chemostat were transferred to the test PBR and grew at the growth condition (30 °C, a PFD of 50 µmol m^{-2} s^{-1}, natural pH, and an aeration rate of 0.3 vvm) with addition of MSX. It is obvious that MSX addition significantly enhanced the excretion of fixed nitrogen by comparison with the control. But the difference among the series of tested MSX concentrations was not very significant at 24h. Until 48h, the difference appeared that the total production of fixed nitrogen from the test in presence of 10 µmol L^{-1} MSX stood out with a production of around 4223 µmol g X^{-1}, followed by 3842, 3535 and 3061 µmol g X^{-1} respectively, produced by the treated cultures in addition of 20, 5 and 30 µmol L^{-1} MSX.

In the light, the release of ammonium by *A. cylindrical* is reported to be maximal at 200 µmol L^{-1} MSX, a concentration which did not affect net CO_2 fixation nor the glycolate excretion, but inhibited the GS activity and the reassimilation of ammonium (Bergman, 1984).

At 24h, there was small amounts of $[NH_3]$g detected for all the tests. During the next 24 hours (from 24h to 48h), the $[NH_4^+/NH_3]$aq of every test culture folded two to three times

and [NH_3]g was also largely produced. Even though the total production of fixed nitrogen at 48h showed significant difference, no severe difference was shown in the production of [NH_3]g, which counted for less than 20 % of the total for all the tests (except control). It can be said that the four MSX concentrations (5, 10, 20, 30 μmol L^{-1}) made no difference in [NH_3]g production under the experimental condition. Then 10 μmol L^{-1} was shown to be the most potential concentration to produce more combined nitrogen.

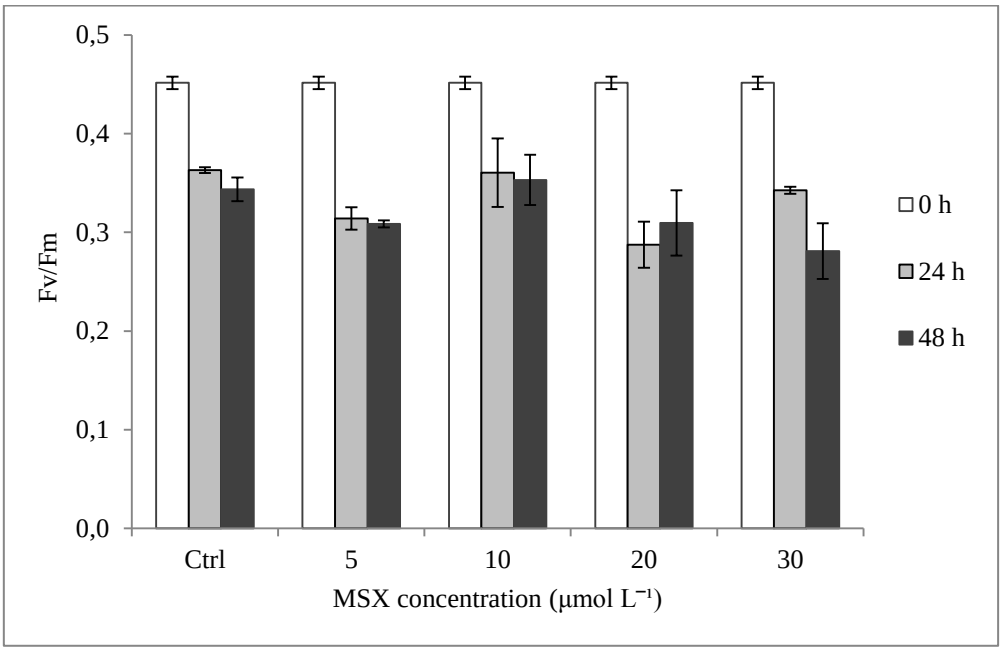


Figure 4−7 b. Photosynthesis activity of the mutant C9 in presence of different concentrations of MSX (T= 30 °C, PFD= 50 μmol m^{-2} s^{-1}, τ=0.3 vvm).

Figure 4−7b presents the photosynthesis characteristics of cultures from all the assays. The Fv/Fm was at the same level with no big difference. Since the excretion of NH_4^+/NH_3 of the assay with 10 μmol L^{-1} MSX treatment was the highest, it confirmed that this MSX concentration was finally to be selected for further study.

4.2.3.2 Effect of photon flux density

Photo production of ammonia by cyanobacteria could be an ideal process, owing to their simple nutritional requirements namely air, water, mineral salts, and light as the main energy source. Nitrogen fixation is a light–dependent process, thanks to PSI activities inside the heterocyt. Besides, light intensity also affects the growth of the vegetative cells under autotrophic condition. Therefore, photon flux density (PFD) might have important influence on nitrogen fixation and ammonia excretion.

Here, four PFD levels, separately, 0 (in dark), 50, 100 and 200 μmol photons m^{-2} s^{-1}, were applied to *A. variabilis* PCC 7937-C9 in presence and absence of MSX for examining their ability for ammonia production (**Figure 4−8a)**.

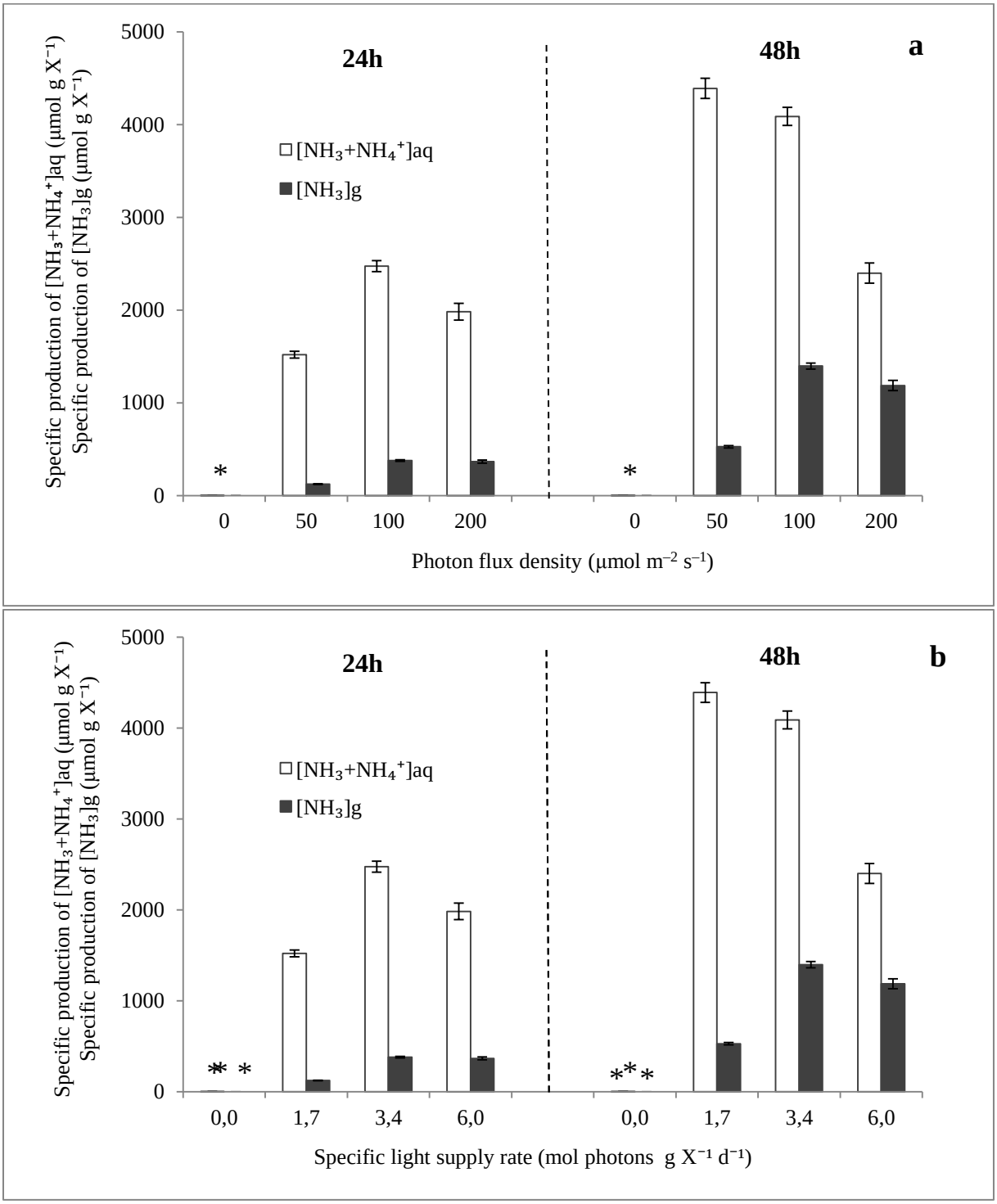


Figure 4−8. Specific production of $[NH_4^+/NH_3]$aq and $[NH_3]$g by the mutant C9 in function of **(a)** PFD levels and **(b)** SLSR levels in presence of 10 μmol L^{-1} MSX (T=30 °C, τ=0.3 vvm)

*Under all the tested PFD levels and SLSR levels, there were little $[NH_4^+/NH_3]$aq or $[NH_3]$g detected in the control groups (without MSX addition).

*When SLSR=0 (in dark), there were no $[NH_3]$g detected even in presence of 10 μmol L^{-1} MSX no matter at 24h or 48h.

As the photons obtained by each cells could be quietly different even the PFD levels are the same but cell concentrations vary, specific light supply rate (SLSR) was also calculated for better understanding the effect of light intensity (**Figure 4−8b)**.

The **Figure 4−8a** presents the specific production of combined nitrogen released in the culture medium and that stripped by the gas phase and trapped in the HCl solution at different PFD levels in presence/absence of MSX.

First of all, no matter at which PFD level, little combined nitrogen was detected in absence of MSX. For the cultures cultivated in dark in presence of MSX, we have confirmed that there was no extracellular NH_3/NH_4^+ detected in culture medium or in the gas phase. By contrast, different specific productions of $[NH_4^+/NH_3]$aq and $[NH_3]$g were obtained at all the tested PFD/SLSR levels.

At 24h, the specific production of $[NH_4^+/NH_3]$aq and $[NH_3]$g obtained by the cultures were maximal at 100 PFD (3.4 SLSR), up to 2475 and 378 μmol g X^{-1}, respectively. Those at 200PFD and 50 PFD were relatively lower. At 48h, the production of $[NH_4^+/NH_3]$aq and $[NH_3]$g at 100 PFD (SLSR=3.4 mol photons g X^{-1} d^{-1}) level were still the highest, but more $[NH_4^+/NH_3]$aq appeared in the culture medium at 50 PFD (SLSR=1.7 mol photons g X^{-1} d^{-1}) than that at 200 PFD (SLSR=6.0 mol photons g X^{-1} d^{-1}) while the production of $[NH_3]$g was in converse. In our culture conditions, 100 PFD (SLSR=3.4 mol photons g X^{-1} d^{-1}) seems to be more suitable for the production of extracellular combined nitrogen, especially $[NH_3]$g.

In a previous study, the effect of light intensity (from 50 to 600 μmol photons m^{-2} s^{-1}) on NH_4-N release by *A. cylindrical* has already been investigated during 2 hours incubation at a cell concentration of 4.8−7.6 μg Chl *a* mL^{-1} (Bergman, 1984). It is suggested that the major source of the ammonium released is the photorespiratory conversion of glycine to serine as the release was stimulated by increase in light intensity. Our result seems to show something different that cultures of *A. variabilis* at 100 μmol photons m^{-2} s^{-1} showed better NH_3/NH_4^+ producing ability. In our study, the cell concentration was around 0.3 g L, which equals to 3.6 μg Chl *a* mL^{-1}, which is at the same level as the study of Bergman, but big differences were those: essentially, different strains were used here and in the other study; secondly, the duration for us was 2 days while theirs was 2 hours; thirdly, 10 μmol L^{-1} MSX was used here which is 1/20 the concentration (200 μmol mL^{-1}) used by Bergman; the last but not least is that gaseous NH_3 was detected and analyzed in our study for the first time. Thus, the results here are not comparable to those reported by Bergman (Bergman, 1984).

At the same time, photosynthesis efficiency of the cells under different PFD/SLSR levels was investigated. From **Figure 4−8c**, it can be seen that the Fv/Fm of the cells in

control groups (without MSX addition) was the highest, around 0.45 during 2 days. The cells treated with MSX but cultivated in dark showed similar photosynthesis efficiency to those at 50 PFD at both 24 h and 48 h, with Fv/Fm above 0.35, which implied that the cultures were less stressed than those at 100 PFD and 200 PFD. This might be the reason why these cells fixed the most dinitrogen from 24 h to 48 h. However the $[NH_3]g$ production at 50 PFD was the lowest after 48h incubation, which was 62.3 % lower than that at 100 PFD and 55.7 % lower than that at 200 PFD. This shows that PFD has influence on dissolved NH_4^+ and NH_4^+/NH_3 equilibrium in the culture medium. For 100 PFD, even though the cells were severely stressed, there was more $[NH_4^+/NH_3]$ aq and $[NH_3]g$ than those at 50 and 200 PFD at 24 h and 48 h, so we primarily considered 100 PFD to be the better one among all the PFDs tested.

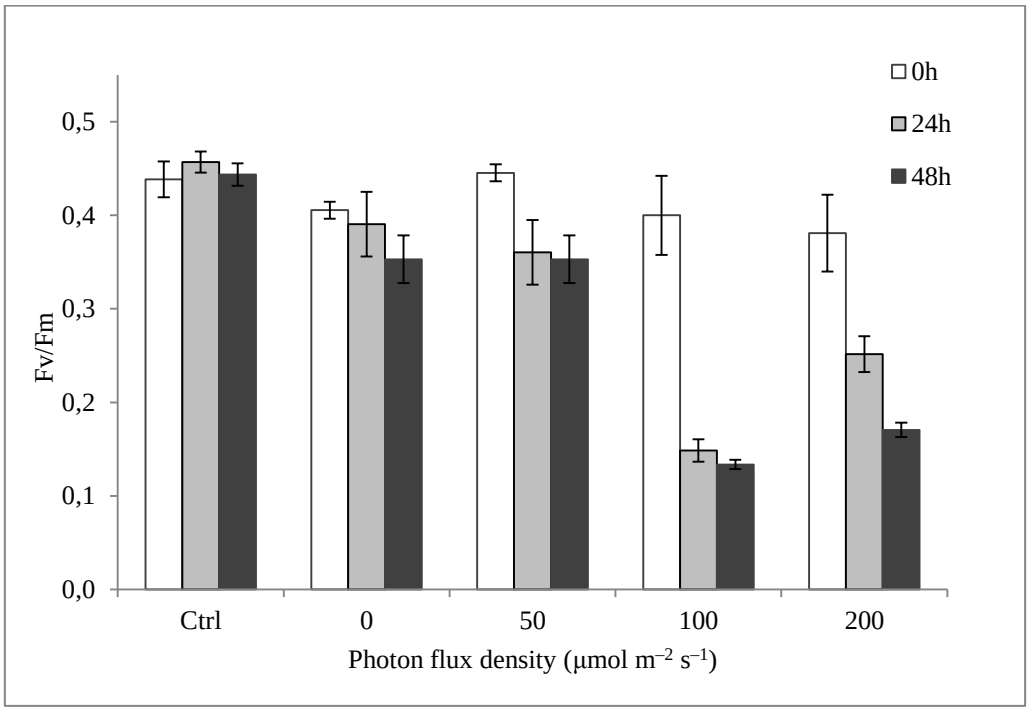


Figure 4−8 c. Photosynthesis activity of *A. variabilis* at different PFD levels in presence/absence of 10 μmol L^{-1} MSX (T=30 °C, τ=0.3 vvm).

Irradiance directly affects the PSII, which produces oxygen, and oxygen can irreversibly inhibit the Nase activity. Respiratory O_2 consumption is thought to be a significant element for protecting Nase from both atmospheric and photosynthetic O_2 (Gallon et al., 1993). When the cells were stressed by strong irradiance, then less oxygen was produced which had less inhibiting impact on the Nase. So, more nitrogen was fixed due to high Nase activity. For 100 PFD, Fv/Fm was shown to be lower than that at 200 PFD and 50

PFD, that's to say, these cells were more stressed than the others. If the stress only came from irradiance, 100 PFD should be higher than that at 200 PFD, but the fact is in opposite. Here, the stress from NH_3 and MSX has to be thought. As it is seen, at 100 PFD, the production of $[NH_3+NH_4^+]aq$ and $[NH_3]g$ were both higher than those at 200 PFD and 50 PFD, this might imply that in the PFD range less than 200 μmol photons m^{-2} s^{-1}, the stress for the cells mainly comes from chemical influence rather than photoinhibition.

4.2.3.3 Effect of temperature

As it is known, temperature has significant influence on enzyme activity, for instance nitrogenase, which catalyzes the nitrogen fixation in heterocyts of *A. variabilis*. Earlier work has shown that high temperature increases the susceptibility of cyanobacterial Nase to inactivation by oxygen (Compaoré and Stal, 2010). There is also report saying that the Nase activity of *A. cylindrical* was totally inhibited at 40 °C when cells were bubbled with air, but little affected in H_2 or argon at this temperature (Gallon et al., 1993). It is indicated that inhibition of N_2 fixation is not due to thermal inactivation of Nase no matter in heterocytous cyanobacteria or unicellular cyanobacteria. Rather, inactivation of N_2 fixation at elevated temperature seemed to be a consequence of increased sensitivity to inhibition by O_2.

In our study, three temperature levels 20°C, 30°C and 40°C were applied to different batch cultures for their potential impact on NH_3/NH_4^+ production,. The elevated temperature 40 °C did not show complete inhibition of nitrogen fixation or ammonia excretion even when cells were bubbled with air. Apparently the production of $[NH_3+NH_4^+]aq$ and $[NH_3]g$ were both the highest either at 24h $[NH_4^+/NH_3]aq$ = 2787 μmol g X^{-1}; $[NH_3]g$= 504 μmol g X^{-1}) or at 48h ($[NH_4^+/NH_3]aq$ = 4690 μmol g X^{-1}; $[NH_3]g$ = 2395 μmol g X^{-1}) (**Figure 4−9a)**. In particular, at 48h, the $[NH_3+NH_4^+]aq$ production at 40 °C was significantly higher than those at 20 or 30 °C. These data illustrated that elevated temperature 40 °C had positive effect on nitrogen fixation and ammonia excretion for the mutant strain C9.

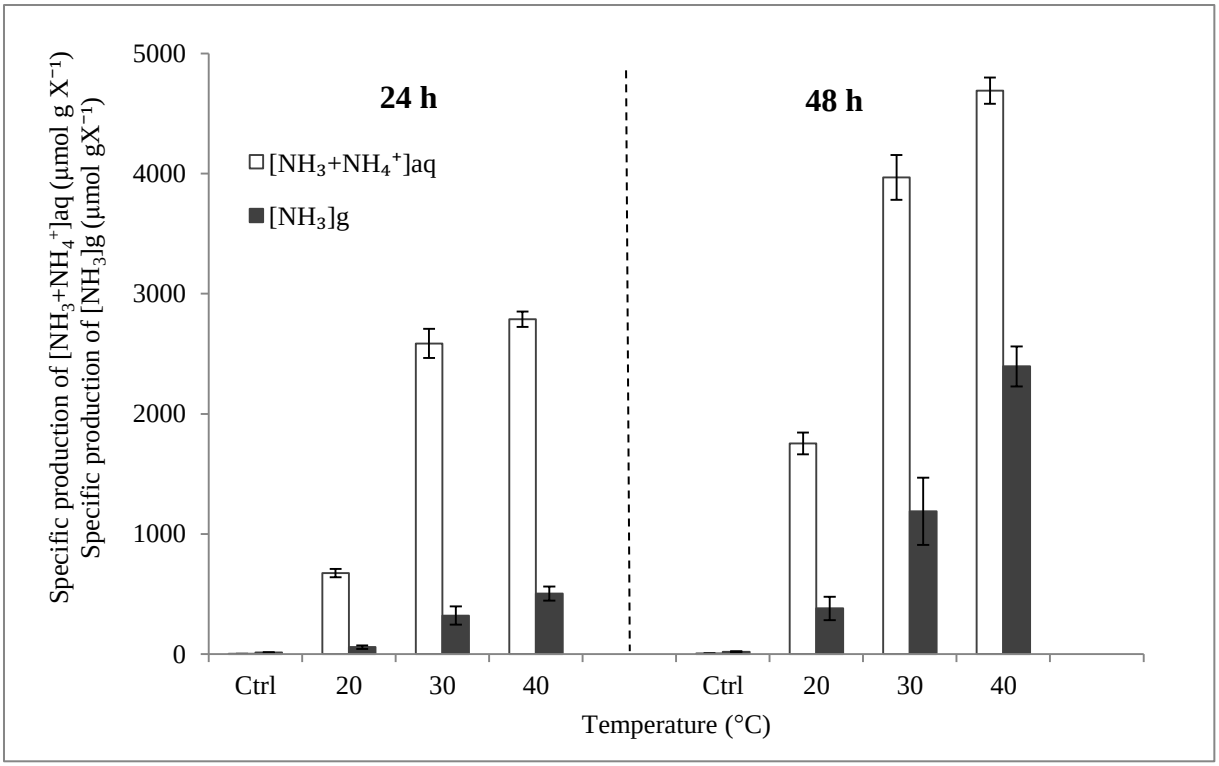


Figure 4−9 a. Specific production of $[NH_4^+/NH_3]$ aq and $[NH_3]$g by the mutant C9 at different temperature levels in presence/absence of 10 µmol L^{-1} MSX (PFD=100 µmol m^{-2} s^{-1}, τ=0.3 vvm)
*Under all the tested T levels, there were little $[NH_4^+/NH_3]$aq or $[NH_3]$g detected in the control groups (without MSX addition).

In terms of $[NH_3]$g, cultures at 40 °C produced 2395 µmol NH_3 g X^{-1} at 48h, which was twice of that at 30 °C (1200 µmol g X^{-1}) and more than six times of that at 20 °C (380 µmol g X^{-1}). As more nitrogen was fixed, it's easy to understand why more ammonia was released to gas phase, because temperature is one of the major influencing factors for NH_4^+/NH_3 equilibrium in solution putting pH aside (Thurston et al., 1979). Additionally, the culture medium was a complex system with various chemicals and cells, all of which would have effect on the solubility equilibrium of $NH_4^{+/}NH_3$.

Figure 4−9b shows the photosynthesis activity of the cultures treated by MSX at different temperatures, namely 20, 30, 40 °C, at 0 h, 24 h and 48 h. In comparison to the control, the Fv/Fm of all the cultures treated by MSX was lower, and decreased as time went on. At the moment when MSX was added, the Fv/Fm for assays at 40 °C was the highest, 0.46, followed by that for 20 °C and 30 °C, respectively, 0.42 and 0.37. 48h later, Fv/Fm of the cells in control group (without MSX) had no variation, but those treated by MSX at 20, 30 and 40 °C dropped from 0.42 to 0.1, from 0.37 to 0.32 and from 0.46 to 0.38, separately. This indicates that MSX addition clearly reduced the photosynthesis activity of *Anabaena* cells. However, in presence of MSX, production of cells at 20 °C were the evidently lower than

those at 30 °C and 40 °C. These could be caused by several reasons: firstly, high temperature supplied more energy for electron transfer chain which enhanced PSII and Fv/Fm; secondly, at higher temperature, more ammonia was excreted and dissolved in the culture medium, which allowed the cultures to assimilate combined nitrogen easier.

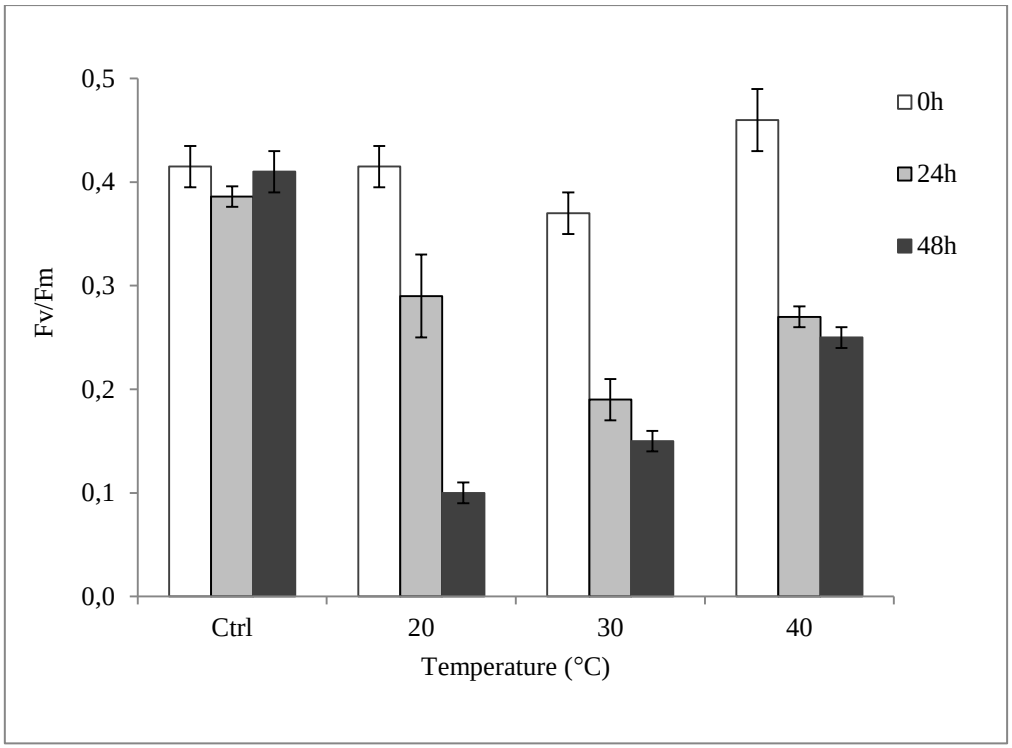


Figure 4−9b. Effect of temperature on the photosynthesis activity (Fv/Fm) of *A. variabilis* in presence/absence of 10 μmol L^{-1} MSX (PFD=100 μmol m^{-2} s^{-1}, τ=0.3 vvm)

Taking both NH_3/ NH_4^+ production and photosynthesis activity (represented by Fv/Fm) in consideration, 40 °C was proven to be the best due to higher NH_3/ NH_4^+ production and higher photosynthesis activity than 20 and 30°C. However, to maintain a temperature of 40°C in a photobioreactor is energy consuming, needing for application a low cost source of heat. Thus, 40 °C will not be applied in the following investigations here, but it's possible to be used in summer or somewhere hot for real application. In addition, cells at 30 °C have shown higher production of both [NH_4^+/NH_3]aq and [NH_3]g than those at 20 °C. Then, it would be better to keep cultures at 30 °C for further study.

4.3.3.4 Effect of the aeration rate

Ammonia production benefits from nitrogen fixation which was catalyzed by Nase, an enzyme very sensitive to oxygen. For nitrogen fixation, the nitrogen generally comes from the atmosphere. Meanwhile, oxygen is also transferred to the culture and has impact on the Nase.

In addition, the solubility and liberation of NH_3 is also affected by gas transfer efficient. So, gas supply must have important influence on the production of combined nitrogen by *A. variabilis*. In this part, air was supplied to the cultures in presence/absence of MSX at a series of aeration rates, 0.3, 0.6, 1.2 and 2.4 vvm (**Figure 4−10)**.

Higher productions of combined nitrogen were obtained when aeration was supplied with aeration at higher rates (**Figure 4−10a**). The highest production of fixed nitrogen reached 10,000 μmol g X^{-1} at 2.4 vvm at 48h, which was, respectively, 109.3 %, 39.0 % and 17.5 % higher than those at 0.3 vvm, 0.6 vvm and 1.2 vvm. This should be mostly thanks to more nitrogen supply to the cells along with higher aeration, because the base were the same for all the assays at the beginning, such as MSX addition, culture volume, the cell state and environmental parameters. Accordingly, limit in nitrogen supply may exist for nitrogen fixation by the mutant strain C9.

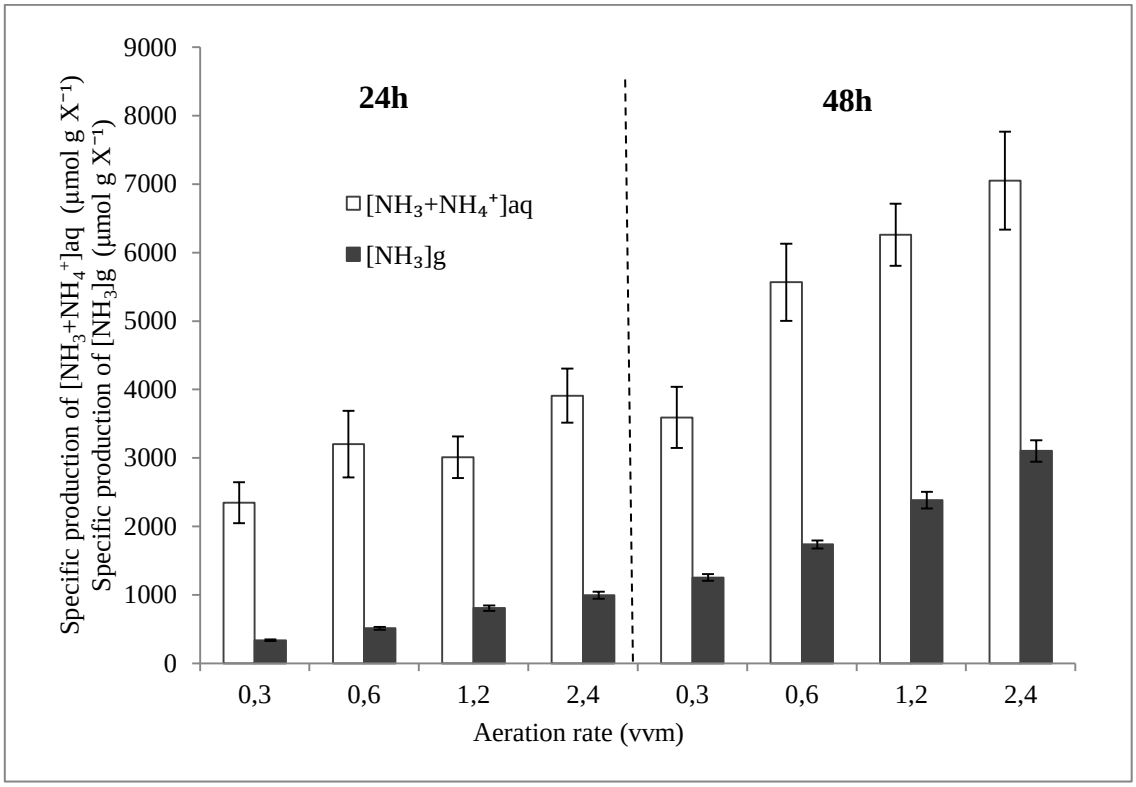


Figure 4−10 a. Specific production of [NH_4^+/NH_3]aq and [NH_3]g by the mutant C9 at different aeration rates in presence of 10 μmol L^{-1} MSX (T=30 °C, PFD=100 μmol m^{-2} s^{-1}).
*Under all the tested τ levels, there were little [NH_4^+/NH_3]aq or [NH_3]g detected in the control groups (without MSX addition).

In **Figure 4−10a**, strong bubbling seems to have enhanced the transfer efficiency of NH_3 from liquid phase to gas phase. Consequently, larger amounts of NH_3 were released to gas phase together with air bubbling. Under this case, the chemical equilibrium between NH_3

and NH_4^+ in the culture medium was displaced, according to the pH value and to the efficiency of the NH_3 stripping. So, more NH_3 was released to gas phase at higher aeration rate. At 48 h, the part of NH_3 released to gas phase counted, 25.8 %, 23.8 %, 27.5 % and 30.6 % of the total amount of fixed nitrogen, separately, at 0.3, 0.6, 1.2 and 2.4 vvm.

We have seen that the aeration rates are related to the overall ammonia gas mass transfer coefficient ($k_L a_{NH_3}$) (**Figure 4−10b**). Then, it is possible to integer for all the duration of these batch cultures the total ammonia trapped in the acid solution and to look for the relation between k_La and these values (**Figure 4−10c**). A relatively good linear relation as shown by the relation:

$$Q_{NH3g} = 0.2525\ k_L a_{NH_3} + 0.0684\ (R^2 = 0.9798)$$

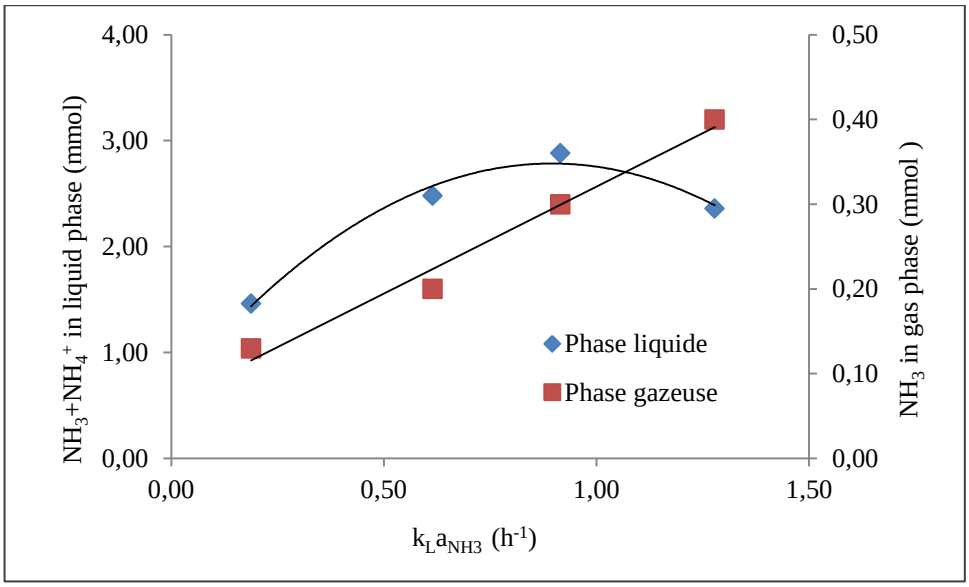


Figure 4−10 b. Effect of $k_L a_{NH_3}$ (h^{-1}) on the global extracellular production of combined nitrogen by cultures of the mutant strain C9 in presence of 10 µmol L^{-1} MSX (T=30 °C, PFD=100 µmol m^{-2} s^{-1}).

The relation between the maximal concentration of $[NH_4^+/NH_3]aq$ with k_La values differs from that of $[NH_3]g$. The lowest value of $[NH_4^+/NH_3]aq$ concentration corresponds to the lowest k_La value. Higher values of k_La seem to have the same impact on accumulation of combined nitrogen in the liquid phase.

The raw data obtained as a function of k_La are presented in the **Figure 4−10b**. The NH_3 accumulation in the acid solution is relatively linear, and the duration of this phase of accumulation is in the range of 100 to 150 hours according to the value of k_La. Then there was no more accumulation. As the solubility of ammonia is very high, a saturation of the acid

solution is not involved. However, we observe that it corresponds to a progressive decrease of the $[NH_4^+/NH_3]aq$ concentration in the liquid phase. This process occurred whatever the k_La value.

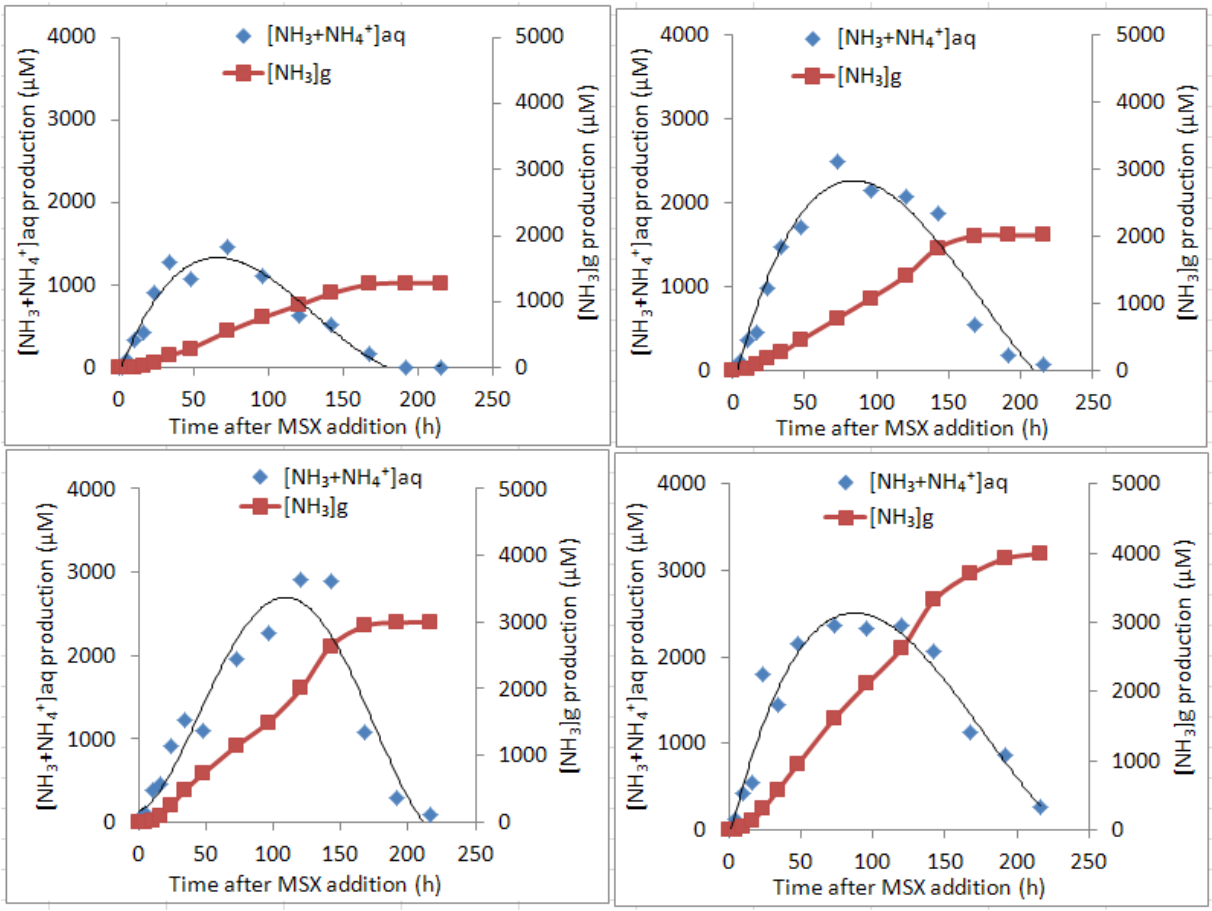


Figure 4−10 c. Kinetics of combined nitrogen accumulation either in the liquid phase or in the gas phase as a function of k_La_{NH3} values in cultures of the mutant strain C9 in the presence of 10 µmol L^{-1} MSX (T=30 °C, PFD=100 µmol m^{-2} s^{-1}).

This decrease of total ammonia and ammonium concentrations in the culture medium could indicate the existence of a threshold of ammonium concentration at which the filamentous cyanobacteria could stop their N_2-fixing activities. Continuous cultures at steady-states could help for characterization of such a process, as shown in the next chapter.

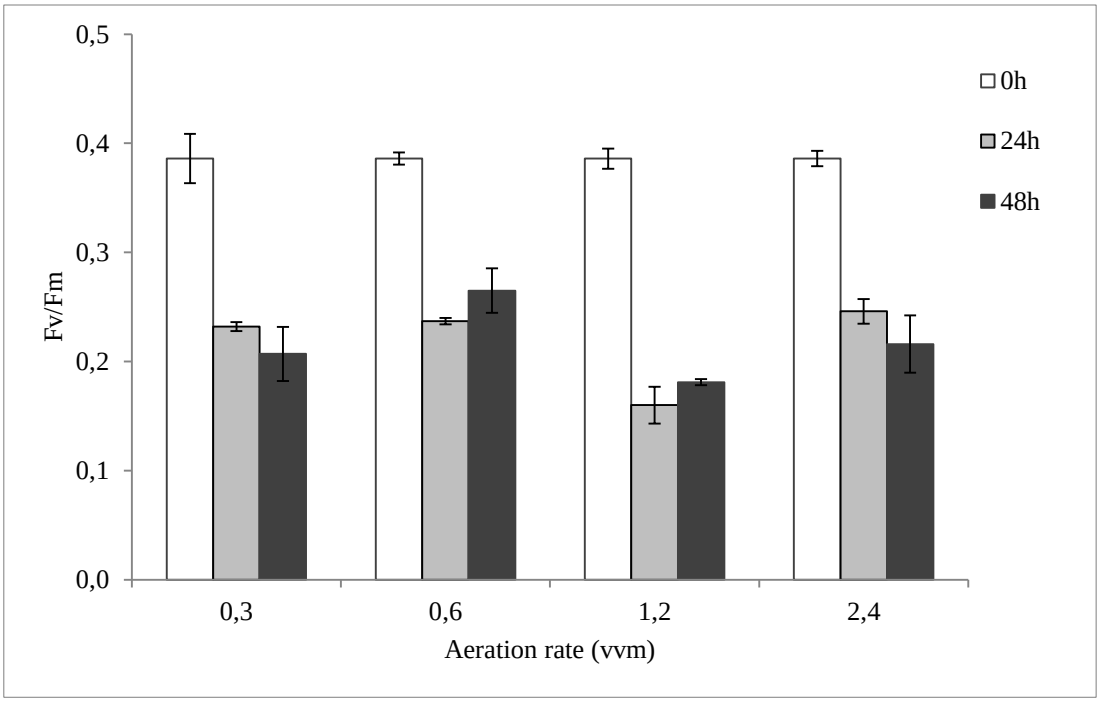


Figure 4−10 d. Photosynthesis activity (Fv/Fm) of cells from C9 cultures at different aeration rates(τ) in presence of 10 μmol L^{-1} MSX at 0 h, 24 h and 48 h (T=30 °C, PFD=100 μmol m^{-2} s^{-1}).

Seeing the photosynthesis characteristic Fv/Fm in **Figure 4−10d**, no significant difference appeared in the photosynthesis activity of the cultures at all the tested aeration rates that all of which decreased around 40 %. In **Figure 4−10a**, combined nitrogen stayed in culture medium varied significantly among different aeration rates, but no significant difference was shown in Fv/Fm in **Figure 4−10d**. This might tell us that little influence of aeration rate occurs on Fv/Fm of the cells even when [NH_4^+/NH_3]aq reached a certain level.

Concerning the photosynthesis efficiency, it is shown that the addition of MSX did not cause much decrease in the Fv/Fm of the cultures at 0 vvm aeration rate at 24 h or even 48 h comparing to that at 0 h. This might be due to the protection of colonies by the cells themselves as they deposited at the bottom when there was no aeration. Surely, MSX addition induced culture pH increase and NH_4^+/NH_3 appearing in the culture medium even at 0 vvm aeration rate. The major difference of the external environments for the assays at 0 vvm from those at 0.3−2.4 vvm was no aeration, so the apparent difference in Fv/Fm could be chiefly caused by lack of aeration rather than high pH or existence of NH_4^+ or NH_3 in culture medium.

4.3 Conclusion

Gaseous NH_3 production from N_2 fixation by *A. variabilis* cultures was determined after that MSX addition induced pH increase in the culture medium, as presumed in our theoretical analysis. The increase of pH could also lead to the equilibrium change of the distribution between NH_3 and NH_4^+, since it depends on the pH of the solution. Among the four MSX concentrations, 10 μmol L^{-1} MSX resulted in the most amount of NH_4^+ and NH_3 at both 24h and 48h. There was no big difference between the excretion of NH_4^+/NH_3 treated by 5 and 20 μmol L^{-1} MSX. PFD also has apparent influence on the production of $[NH_3+NH_4^+]aq$ and $[NH_3]g$. At 100 PFD, the $[NH_4^+/NH_3]aq$ and gaseous NH_3 were both the higher than those at 200 PFD and 50 PFD, this implies the stress for these three PFDs came from NH_3 toxicity rather than irradiance. So, in the tested PFD ranges, the stress for the cells seemed mainly come from chemical influence, whilst irradiance gave more stress to the cells when PFD is higher than 200. In terms of another important parameter, temperature, 40 °C was proven to be the best among the tested range due to higher NH_4^+/NH_3 production and higher photosynthesis activity. However, higher temperature is more energy consumed for managing and maintaining, while 20 and 30 °C could be easily achieved at ambient condition. It would be better to keep cultures at 30 °C for further investigation, but 40°C can also be considered for application, for example in summer or somewhere hot on the earth. Moreover, higher aeration rate is shown to be advantageous for the production of $[NH_3]g$. Without bubbling, NH_3 could not be released to the gas phase. Then, aeration of the culture increasing the exchange of NH_3 between liquid phase and gas phase is essential for gaseous NH_3 production.

CHAPTER V Ammonia production by *A. variabilis* PCC7937-C9 in continuous cultures

5.1 Introduction

Even though people have done a lot of works on bio-production of nitrogen bio-fertilizer with the help of cyanobacteria, low ammonia productivity or instability of mutant are still problems to be solved. Another way could be to convert the biomass of cyanobacteria to biogas or ammonium sulfate as proposed by Razon (2012). However, low biomass productivities restricted this application. Among all the existing literatures, no study concerning continuous bio-production of ammonium was found.

In our first part of study, the biomass productivity of the wild-type strain of *A. variabilis* was shown to be improved up to 0.80 g L^{-1} d^{-1} under diazotrophic condition by supplying with CO_2 through pH regulation. According to our analysis, it seems possible to further improve the biomass productivity.

Another exciting observation is that gaseous NH_3 produced by the MSX resistant strain C9 was detected and successfully stripped by the gas phase and trapped with acid solution. Such way could simplify the valorization of fixed nitrogen produced by cyanobacteria to a certain extent comparing to former studies. The production of gaseous NH_3 by *A. variabilis* was proved to be closely related to culture pH.

The aim of the next experiments was to confirm the results obtained in batch cultures by growing the mutant strain in continuous cultures. Thanks to steady state cultures, the study was focused on the extracellular NH_3/NH_4^+ production by free cells of *A. variabilis* in the presence of MSX. The question was to characterize the impact of MSX treatment on the biomass and extracellular combined nitrogen production. Indeed, negative impacts of MSX on the growth of bacteria and plants have been widely reported (Lin and Kao, 1996). Since the MSX is toxic, it is difficult to sustain prolonged NH_3 production in presence of MSX in bioreactors (Kerby et al., 1986). The death of *Anabaena* even could be induced by the toxicity of extracellular NH_3 (Britto and Kronzucker, 2002).

Two means of MSX addition were investigated. The kinetics of production of $[NH_4^+/NH_3]$aq and $[NH_3]$g by continuous cultures with pH regulated at 6.8 and 8.8 was also studied.

5.2 Results and Discussion

To further highlight the effect of pH on the production of gaseous NH_3, a continuous culture of *A. variabilis* PCC 7937-C9 was started in a 0.7 L PBR with constant pH regulation. All the parameters were fixed, i.e. irradiance (110 µmol photons m^{-2} s^{-1}), temperature (30 °C), aeration rate (0.3 vvm) and $BG11_o$ feed medium at a dilution rate of 0.3 d^{-1}. The kinetics of

biomass, [NH_3]g and [NH_4^+/NH_3]aq production were determined. In addition, the cell viability of present mutant strain in presence of MSX at each state was also analyzed.

5.2.1 Comparison of pulsed and continuous injection of MSX

For our study, MSX addition is essential. In order to find the better method for introducing MSX into a continuous culture, two different means were attempted, i.e. pulse method (injection MSX into the culture frequently, 10 μmol L^{-1} in the culture) or continuous method (injection MSX into feeding medium, 10 μmol L^{-1} in feeding medium). In absence of cyanobacteria, the residual MSX concentration could be estimated as the following equations:

$$\frac{dMSX_t}{dt} = D \cdot (MSX_0 - MSX_t)$$

$$MSX_t = MSX_0 - e^{-D \cdot t + lnMSX_0}$$

As shown in **Figure 5−1,** the residual MSX concentration gradually decreased after pulse injection of MSX into the culture, while it increased when previously injected into the feeding medium.

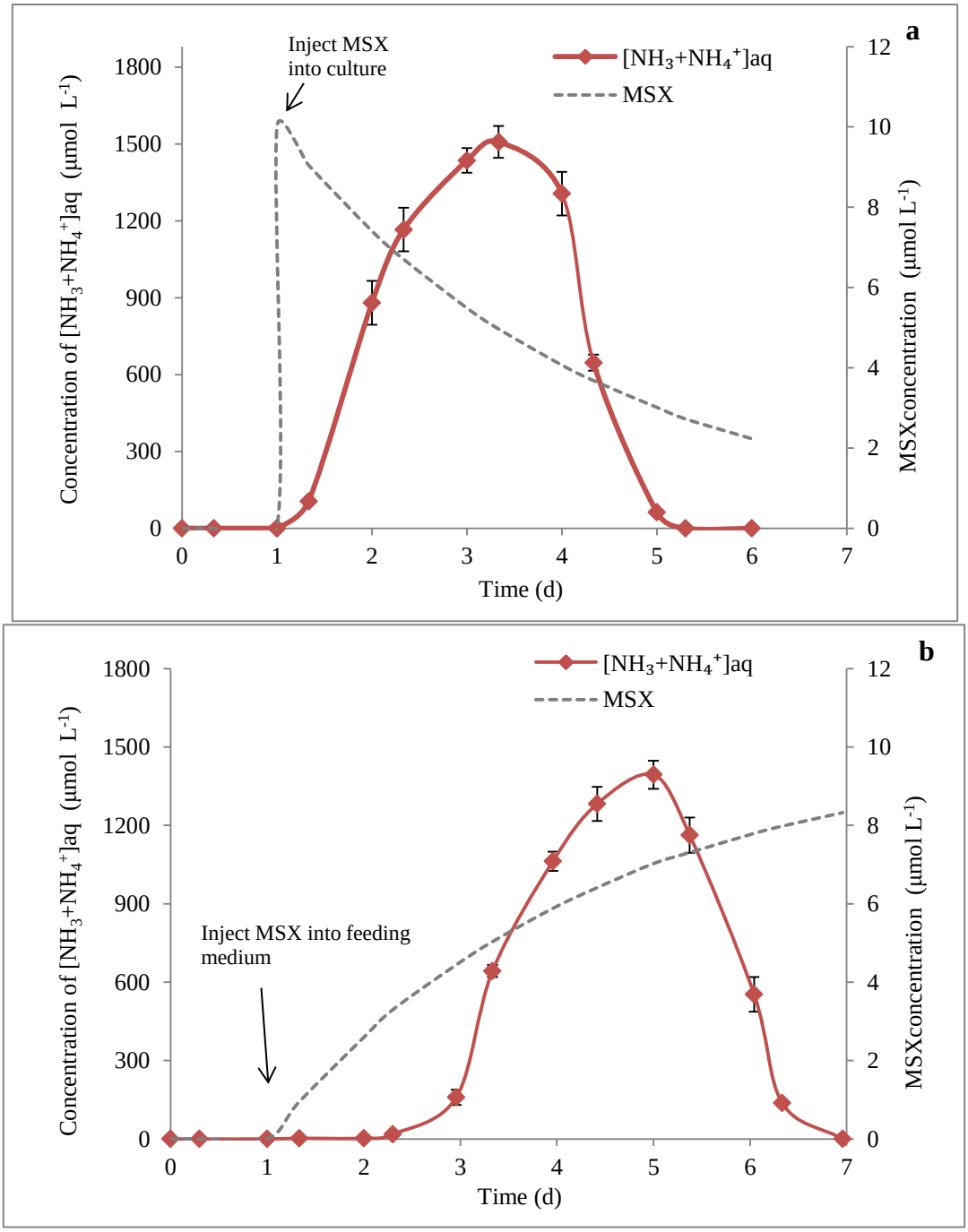


Figure 5−1. Kinetics of actual $[NH_3+NH_4^+]aq$ concentration and simulated MSX concentration through **(a)** pulse injection into culture or **(b)** continuous feed from culture medium in continuous cultures of the mutant strain C9.

A little difference occurred in the $[NH_4^+/NH_3]aq$ production with different MSX adding means. $[NH_4^+/NH_3]aq$ started to appear in the culture medium in a short time after the injection of MSX into cultures as it is shown in **Figure 5−1a**, whereas there were about 1.5 days' delay for the appearance of $[NH_3+NH_4^+]aq$ by continuous MSX injection through the feeding medium (**Figure 5−1b);** this delay is also related to the dead volume. It appeared that a low MSX concentration, i.e. less than 3.0 µmol L^{-1}, in the culture could be not enough for

$[NH_4^+/NH_3]$aq production. However, whatever the injection methods, **Figure 5−1 (a)** and **(b)** show the same tendency in $[NH_3+NH_4^+]$aq, that when $[NH_4^+/NH_3]$aq concentration reached about 1.5 mmol L^{-1}, the $[NH_4^+/NH_3]$aq concentration started to decrease, eventually to 0. The process could last for about 5 days. Therefore, it seems that the activity of Nase was inhibited and cells started to uptake $[NH_4^+/NH_3]$aq from the culture medium, as already observed in batch cultures, as a function of k_La.

If we choose injection MSX into the culture for long time continuous culture, it will be needed to inject MSX every 4 days, in case of MSX washing out. Then, injection MSX into feeding medium could be better for long term continuous culture, even though there is a 1.5–day delay.

5.2.2 Production of $[NH_3+NH_4^+]$aq and $[NH_3]$g with pH regulated at 6.8

In order to characterize the kinetics of growth, $[NH_4^+/NH_3]$aq and gaseous NH_3 production of the mutant strain C9, a relative long term continuous culture with two cycles was carried out with injection MSX into the feeding medium when the cells reached a steady state.

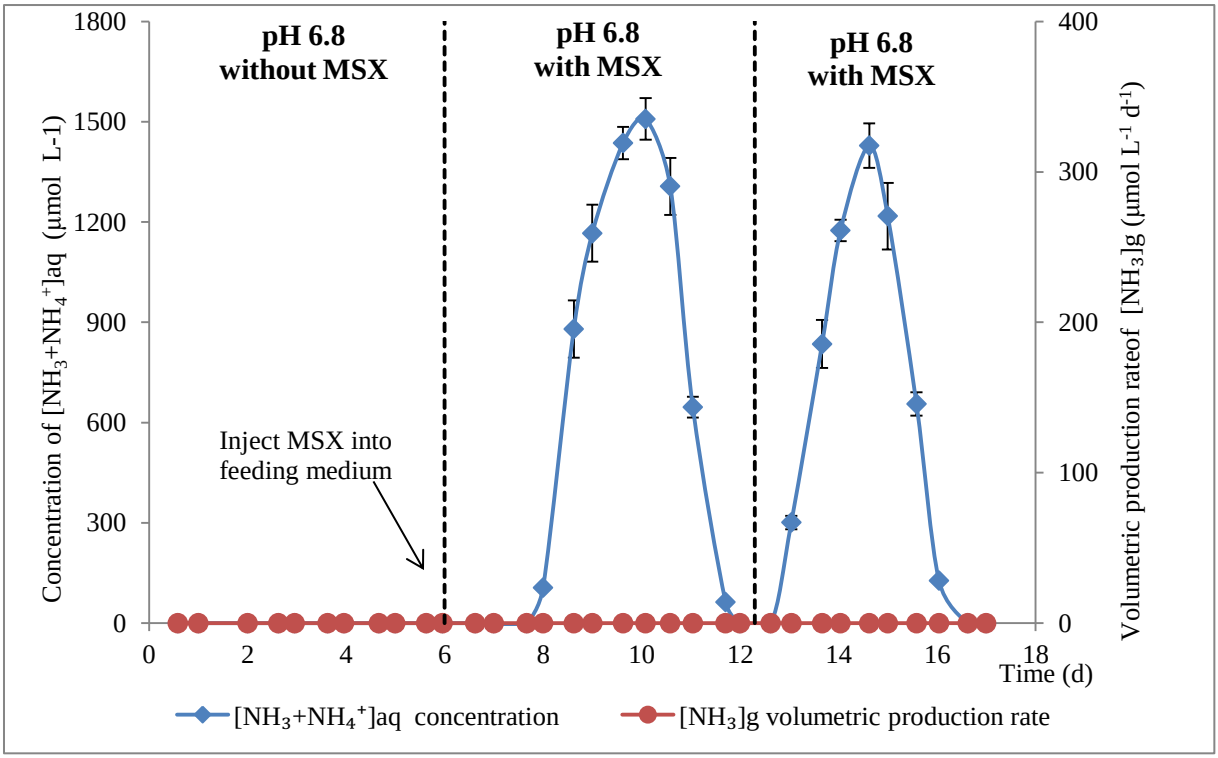


Figure 5−2. Kinetics of $[NH_4^+/NH_3]$aq concentration and $[NH_3]$g production rate by continuous cultures of the mutant strain C9 with pH controlled at pH 6.8 (T= 30°C, PFD=110 µmol m^{-2} s^{-1}, τ=0.3 vvm).

As shown in **Figure 5−2**, there was no $[NH_4^+/NH_3]$aq or $[NH_3]$g observed when culture pH was controlled at pH 6.8 without MSX addition. Moreover, no $[NH_3]$g was produced at this pH value in the presence of MSX. However, 1.5 days after the injection of MSX into the feeding medium, $[NH_4^+/NH_3]$aq was detected and gradually increased to 1.5 mmol L^{-1}. Then, decrease in $[NH_4^+/NH_3]$aq concentration started in the following period, till undetectable. Moreover, short term after the first cycle that $[NH_4^+/NH_3]$aq concentration decreased to undetectable, $[NH_4^+/NH_3]$aq was detected again. Interestingly, the second cycle showed almost the same tendency as the first one with a peak of 1.5 mmol L^{-1} $[NH_4^+/NH_3]$aq concentration. This indicates that the excretion of NH_3 from cells to the culture medium was inhibited, which could be due to the decrease of Nase activity at high $[NH_4^+/NH_3]$aq concentration (≈ 1.5 mmol L^{-1}). In accord, Ernst *et al* (1990a) has already found that supply of NH_4^+ to *A. variabilis* previously grown under diazotrophic conditions, can slow down the Nase activity. Indeed in the cyanobacteria *A. cylindrica*, NH_4^+ at a concentration of 1.0 mmol L^{-1} could completely inhibit Nase activity as it has also been reported (Ohmori and Hattori, 1974). Automatic return to $[NH_4^+/NH_3]$aq production may reflect a disinhibition of Nase. Such kinetic seems in line with the observation of Kleiner (1975) that decrease in Nase activity by the rise in the extracellular NH_4^+ level is reversible. Therefore, even if Nase is inhibited by high $[NH_4^+/NH_3]$aq concentration at pH 6.8, it could reactivate and restart to biosynthesize NH_3 to continue the second cycle, when $[NH_4^+/NH_3]$aq concentration decreased to an undetectable level. A lower threshold value of NH_4^+ for stimulation of Nase activity and a higher threshold value of NH_4^+ for inhibition of Nase activity. The well-known toxicity of high ammonia concentration cannot be totally ruled out.

In present study, the mutant strain C9 was obtained by double random mutagenesis treatments with EMS (Bui et al., 2014). The photosynthetic activity index, Fv/Fm, was not decrease with the increase of MSX concentration in the culture showing no stress of MSX to the cells (**Figure 5–3**). In addition, that biomass concentration increased from 0.6 g L^{-1} to 0.9 g L^{-1} from steady state in absence of MSX to the condition in presence of MSX, demonstrated the tolerance of this mutant strain to MSX (**Figure 5–4**). Then according to these results obtained in continuous cultures, the MSX treatment not only induced ammonia/ammonium leakages but also an increase in the biomass productivities. Such property could involve a stimulation of the Nase activity in this experimental condition.

Moreover, $[NH_4^+/NH_3]aq$ specific production (µmol g X^{-1}) showed the same tendency with a first and a second cycle. Therefore, it confirmed that Nase inhibited by high $[NH_4^+/NH_3]aq$ concentration could be reactivated, when culture pH was regulated at 6.8.

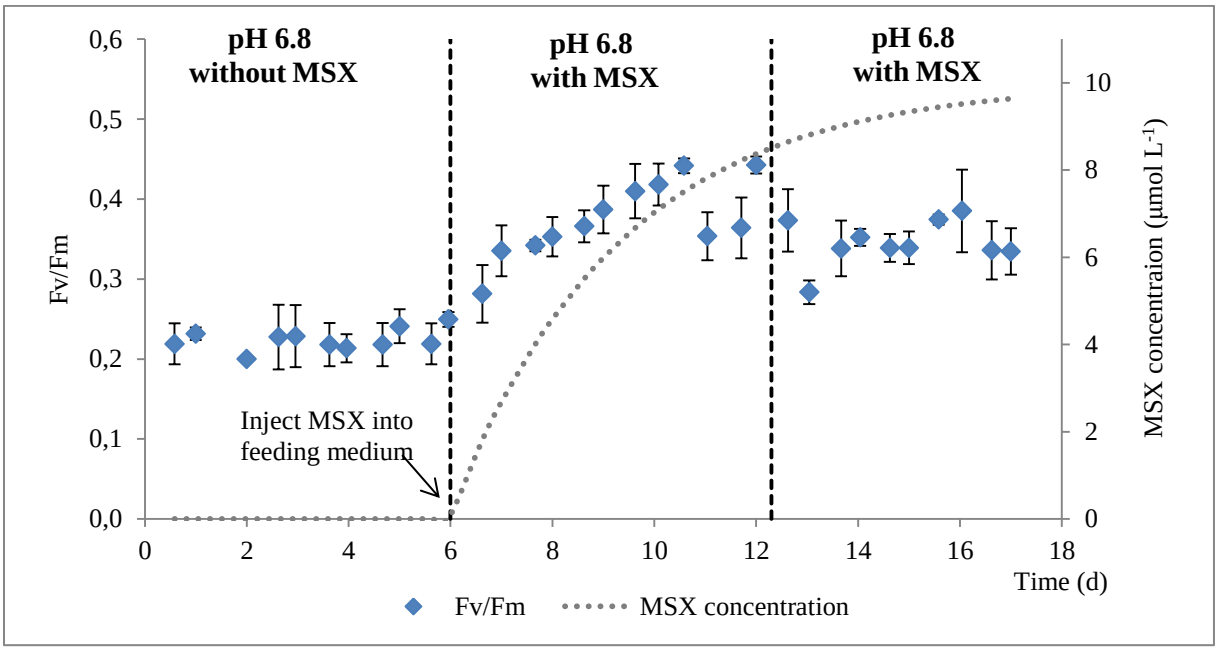


Figure 5–3. Kinetics of photosynthesis efficiency (Fv/Fm) and simulated MSX concentration in continuous culture of the mutant strain C9 with pH regulation set at pH 6.8 (T= 30°C, PFD=110 µmol m^{-2} s^{-1}, τ=0.3 vvm).

Figure 5–4 shows that whatever the MSX concentration, from 0 to almost 10 µmol L^{-1} at pH 6.8, no gaseous NH_3 is produced. If the hypothesis that Nase would be completely inhibited when $[NH_4^+/NH_3]aq$ concentration reached around 1.5 mmol L^{-1}, it would take around 3.3 days from the $[NH_4^+/NH_3]aq$ concentration peak to an undetectable level without $[NH_3]g$ production, taking the dilution rate 0.3 d^{-1} in consideration. However, this period was 2 days at pH 6.8. Considering the biomass concentration for extracellular NH_3 production, it's clear that biomass production increased (**Figure 5–4**). This could have proved that part of $[NH_4^+/NH_3]aq$ was consumed by cells for biomass production at pH 6.8, which shortened the decreasing period. From **Figure 5–3**, high photosynthesis efficiency also showed an evidence for cell growth.

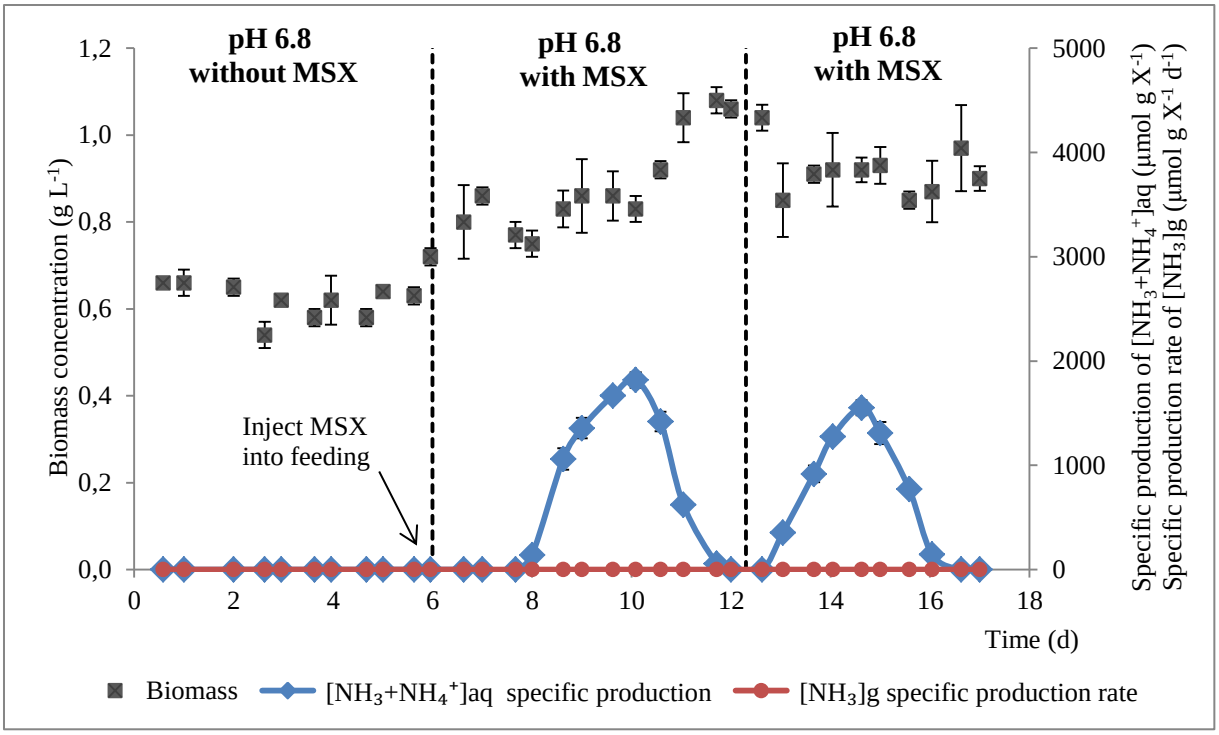


Figure 5–4. Kinetics of biomass concentration, specific production of $[NH_3+NH_4^+]aq$ and $[NH_3]g$ by continuous culture of the mutant strain C9 with pH controlled at 6.8 (T= 30°C, PFD=110 µmol m^{-2} s^{-1}, τ=0.3 vvm).

5.2.3 Ammonia production with two pH set values

Another investigation on the production of extracellular $[NH_4^+/NH_3]aq$ and $[NH_3]g$ by continuous culture was carried out by changing the pH set value, from 6.8 to 8.8. The goal to confirm the pH effect on $[NH_3]g$ production. When biomass concentration was at steady state in absence of MSX at pH 6.8, no $[NH_4^+/NH_3]aq$ or $[NH_3]g$ was detected. Then, MSX was added into the feeding medium at a final concentration of 10 µmol L^{-1}. As expected, $[NH_3+NH_4^+]aq$ gradually increased, whereas there was still no $[NH_3]g$ produced (**Figure 5–5**).

According to our previous result from batch cultures that an increase in pH to high level around 9.0 by pulse regulation could promote $[NH_3]g$ production, pH of the continuous culture was scheduled to be regulated to 8.8 to see if high constant pH condition can enhance the production of $[NH_3]g$ or the duration of $[NH_3]g$ production. From **Figure 5–5**, it could be seen that $[NH_4^+/NH_3]aq$ concentration showed similar tendency as that at pH 6.8, and $[NH_3]g$ production occurred as expected when pH was regulated to 8.8.

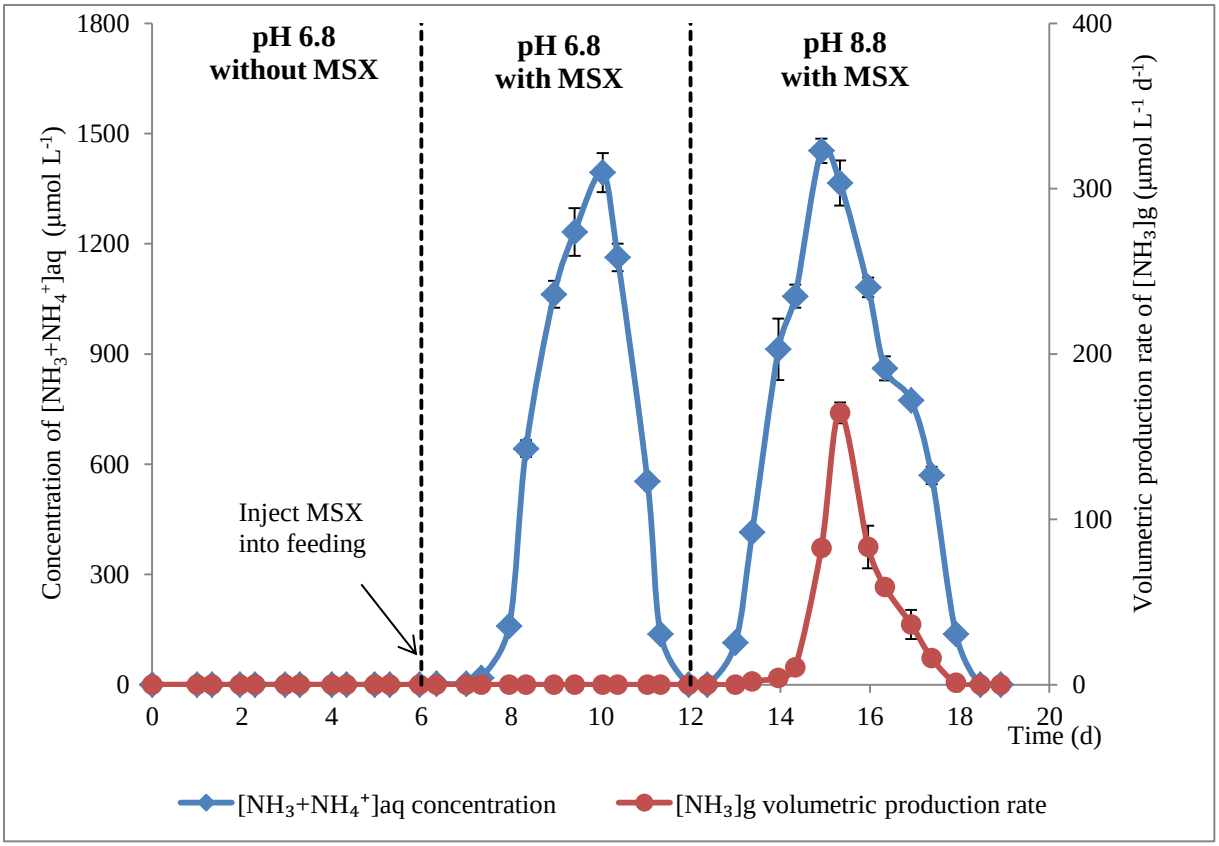


Figure 5–5. Kinetics of actual $[NH_3+NH_4^+]aq$ concentration and volumetric production rate of $[NH_3]g$ in continuous culture of the mutant strain C9 from pH 6.8 to 8.8 (T= 30°C, PFD=110 µmol m^{-2} s^{-1}, τ=0.3 vvm).

The **Figure 5–6** shows that the maximal $[NH_3+NH_4^+]aq$ specific productions were 4505 µmol g X^{-1} at pH 8.8 against 2534 µmol g X^{-1} at pH 6.8 ; the difference is statistically significant ($P<0.05$). However, even with a continuous regulation of the pH at 8.8, a rapid decrease in $[NH_4^+/NH_3]aq$ concentration (**Figure 5–5** and **Figure 5–6**) appeared from the point of 1.45 mmol L^{-1} $[NH_4^+/NH_3]aq$, during a decreasing period about 3.5 days from day 14.9 to day 18.5 with $[NH_3]g$ production. During the same period, the cell concentration decreased 0.43 g L^{-1} to 0.21 g L^{-1} (day 116.3), then increased to 0.30 g L^{-1}, which increased at an average rate of 0.041 g L^{-1} d^{-1}, while at pH 6.8, the biomass concentration increased at an average rate of 0.33 g L^{-1} d^{-1}, which could be calculated from day 6.0 (0.45 g L^{-1}) to day 12.0 (0.67 g L^{-1}). Similar reports have said that pH could affect the $[NH_4^+/NH_3]aq$ uptake by cells, e.g. cyanobacterium *Anacystis nidulans* possesses two NH_4^+ uptake systems with operative pH separately 7.4 and 5.2 (Kashyap and Singh, 1985). Otherwise, it has also been documented that no optimum is observed between pH 5.0 and 9.0 for NH_3 movement through cell wall of *A. azollae* (Zimmerman and Boussiba, 1987). In present study, it seems present mutant *A. variabilis* PCC 7937-C9 could uptake NH_4^+ at pH 6.8 and 8.8.

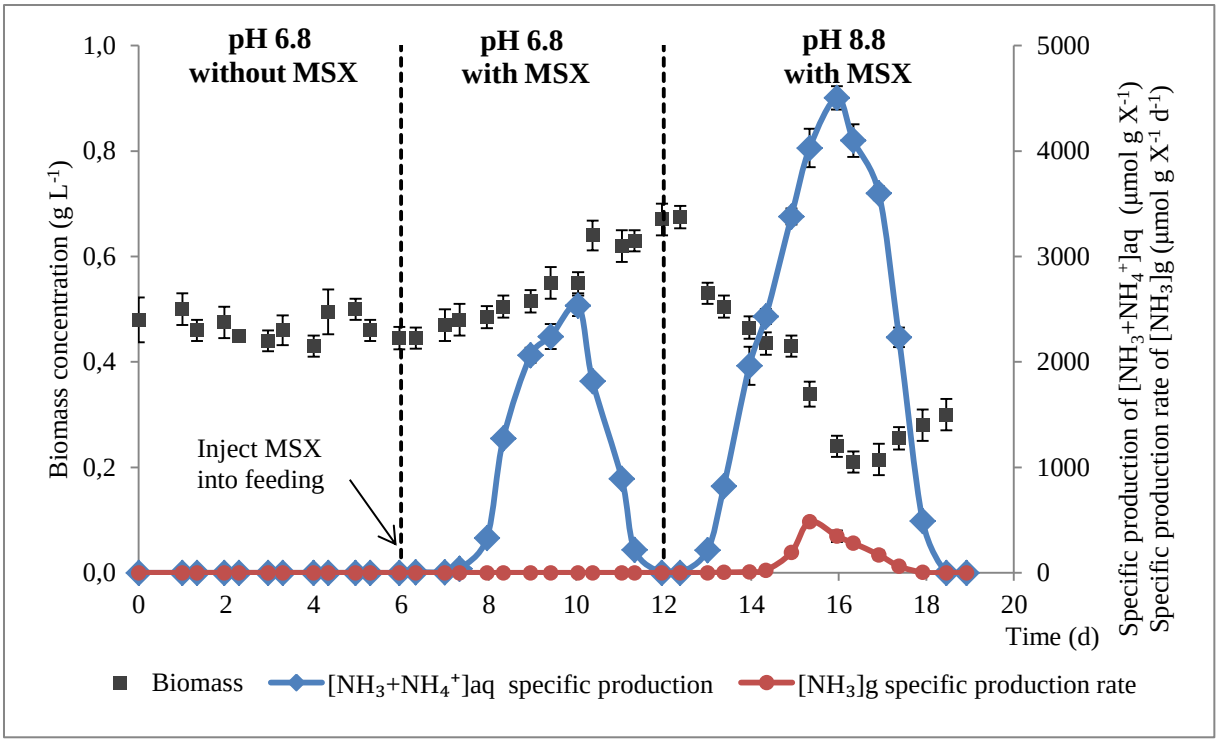


Figure 5−6. Kinetics of biomass concentration, $[NH_3+NH_4^+]aq$ specific production and $[NH_3]g$ specific production rate in continuous culture of the mutant strain C9 from pH 6.8 to 8.8 (T= 30°C, PFD=110 µmol m^{-2} s^{-1}, τ=0.3 vvm).

Moreover, as expected, gaseous NH_3 appeared at pH 8.8 and was produced with both maximal specific production rate and maximal volumetric production rate at maximal $[NH_4^+/NH_3]aq$ point. This confirmed that gaseous NH_3 production rate depends on $[NH_4^+/NH_3]aq$ concentration as demonstrated in batch cultures. Nevertheless, $[NH_3]g$ production rate decreased with the decrease of $[NH_4^+/NH_3]aq$ and after a 6–day $[NH_3]g$ production, the culture stopped producing gaseous NH_3, as the cycle occurred at pH 6.8 (**Figure 5−5** and **Figure 5−6**).

As shown in **Figure 5−5** and **Figure 5−6,** the increase of $[NH_4^+/NH_3]aq$ concentration stopped at round 1.5 mmol L^{-1}, whatever the pH condition at 6.8 or 8.8. The decrease of $[NH_4^+/NH_3]aq$ concentration also confirmed that $[NH_4^+/NH_3]$ produced by *A. variabilis* C9 could be consumed by itself at both pH 6.8 and pH 8.8. Also, present results seem to indicate that Nase could be inhibited at pH 6.8 by high $[NH_4^+/NH_3]aq$ concentration could be reactivated at pH 8.8 when $[NH_4^+/NH_3]aq$ concentration decreased to an undectable level. Consequently, further investigations are necessary to find the conditions for permanent activation of Nase activity, avoiding inhibition by the extracellular NH_4^+, for sustained continuous gaseous NH_3 production.

Figure 5–7 shows that the value of Fv/Fm and ETR_{max} as photosynthetic activity was significantly higher at pH 6.8 in presence with MSX than that in absence with MSX ($P<0.05$). However, at pH 8.8 with MSX, the biomass production, Fv/Fm and ETR_{max} significantly decreased comparing to that at pH 6.8. There are some studies relating to growth and pH conditions. For the strain *A. ambigua* cultivated in BG11 medium , pH 7.0 is shown to be the best (Reddy et al., 2013). Growth of five *Azolla* populations is comparable from pH 5.0 to pH 8.0, but decreased at pH 9.0 (Peters et al., 1980). Because controlling pH at 8.8 needed to inject NaOH, high Na^+ concentration reaching 1150 mg L^{-1} was observed at the late stage of continuous culture at pH 8.8. Then, the high salinity could be also a reason for limiting the cell growth. At 30 °C, there are 26.59% CO_2(aq) and 73.35% HCO_3^- at pH 6.8 and no CO_2(aq) and about 98% HCO_3^-.at pH 8.8,which might have some effect on the CO_2 fixation (Fv/Fm) of the cells and biomass production or NH_3/NH_4^+ production. Anyhow, the reasons influencing the growth of present mutant strain *A. variabilis* PCC 7937-C9 at high pH condition could be furtherly investigated.

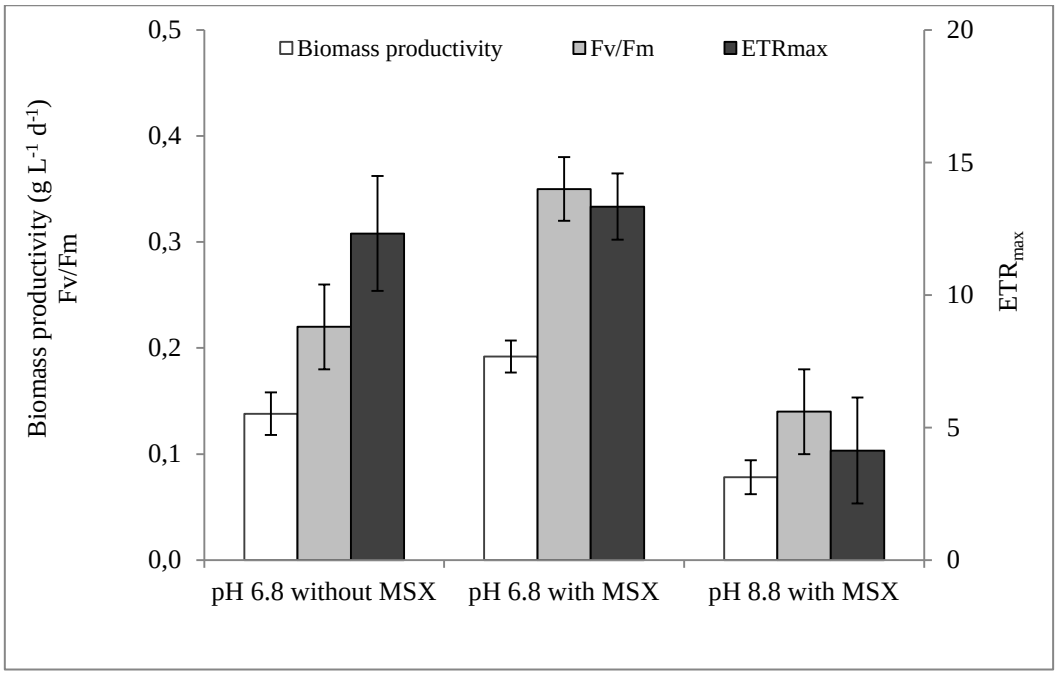


Figure 5−7. Biomass productivity, Fv/Fm and ETR_{max} at different pH and MSX state in continuous cultures of the mutant strain C9.

Whatever, the kinetic data in continuous culture from pH 6.8 to 8.8 confirmed our hypothesis that no production of gaseous NH_3 at low pH, because $[NH_3]$aq only rates 0.6 % of $[NH_4^+/NH_3]$aq at pH 6.8 according to NH_4^+/NH_3 equilibrium, while the ratio is 38.8% at pH 8.8.

5.2.4 Maximal production of extracellular NH_3/NH_4^+ at pH 6.8/8.8 in continuous cultures

From **Table 5−1**, it is seen that more nitrogen was fixed at higher pH mainly thanks to gaseous NH_3 stripping from the culture medium. No matter at which pH, the maximal $[NH_4^+/NH_3]$aq concentration was about of the same order of magnitude, around 1.5 mmol L^{-1}, which was preliminarily considered as the critical level inhibiting Nase activity.

Table 5−1
Production of each part of combined nitrogen fixed by C9 in continuous cultures with controlling pH at 6.8 and 8.8 (unit: µmol L^{-1}).

	pH 6.8	pH 8.8
$[NH_4^+/NH_3]$ aq	1393.8±53.5	1453.3±33.2
$[NH_3]$aq	13.2±1.2	755.7±25.7
$[NH_4^+]$aq	1380.1±52.7	697.6±23.6
$[NH_3]$g	0	237.5±6.3
$[NH_4^+/NH_3]$total	1394±53.5	1690±39.5

τ =0.2 vvm; T=30 °C; PFD=110 µmol m^{-2} s^{-1}; MSX concentration=10 µmol L^{-1}.

Table 5−2 highlights the fact that the present work is the first study dealing with the ammonia production in the gas phase ($[NH_3]$g). The maximal productions of $[NH_4^+/NH_3]$aq and $[NH_3]$g in present study are compared to those in previous studies in the presence of MSX. We have obtained the highest values for production of $[NH_4^+/NH_3]$aq by free living cells in batch cultures, while the production by continuous cultures was just at the same level as the others. From this table, it can also be seen that immobilization could help improve the production of combined nitrogen. Then, this could be considered in further investigations.

Table 5−2
Comparison of maximal production rates of $[NH_3+NH_4^+]aq$ and $[NH_3]g$ by the mutant strain C9 and those in previous studies in presence of MSX.

Species	Cell state	MSX ($\mu mol\ L^{-1}$)	$[NH_4^+/NH_3]aq$	$[NH_3]g$	$[NH_4^+/NH_3]total$ ($\mu mol\ mg\ Chl^{-1}\ h^{-1}$)	Cultivation model	Reference
A. variabilis PCC7937-C9	FL	10	4.7	1.7	6.4	C	this study
A. variabilis PCC7937-C9	FL	10	12.3	5.4	17.7	B	this study
A. varibilis PCC7937-C9	FL	10	4.9	n.a.	4.9	B	Bui *et al.*, 2014
A. varibilis PCC7937-WT	FL	10	3.5	n.a.	3.5	B	Bui *et al.*, 2014
A. siamensis B 11.82 SS1	FL	500	8.7	n.a.	8.7	B/C	Thomas *et al.*, 1991
A. siamensis B 11.82 SS1	FL	500	8.0	n.a.	8.0	B	Thomas *et al.*, 1990
Anabaena 27893	Im	10	40	n.a.	40	B	Kerby *et al.*, 1986
A. cylindrica	Im	50	0.4	n.a.	0.4	B	Jeanfils and Loudeche, 1986
A. azollae	Im	50	6.7	n.a.	6.7	B	Brouers and Hall, 1986
Anabaena ATCC 27893	Im	10	8.5	n.a.	8.5	B	Musgrave *et al.*, 1982
A. azollae	Im	50	7.4	n.a.	7.4	B	Shi *et al.*, 1987
Mastigocladus laminosus 1447/l	Im	50	4.0	n.a.	4.0	B	Brouers and Hall, 1986

FL=Free living; Im=Immobilized; B=Batch culture; C=Continuous culture; M=Mutant; n.a.=Not analyzed.

However, now we know that we have to take in account the fact that ammonia excreting cyanobacteria would create pH and oxygen gradients within the matrix of immobilization, such as calcium alginate beads. Such micro-environmental conditions will be interesting to investigate, which could explain the relatively high values of dissolved ammonium/ammonia published, but the most interesting information will be to quantify the rates of gaseous ammonia production by such immobilized cells.

5.3 Conclusion

The kinetic process of $[NH_4^+/NH_3]aq$ and $[NH_3]g$ production was investigated in continuous cultures with controlling pH. The kinetic data in continuous cultures from pH 6.8 to 8.8 confirmed our hypothesis that pH affects the production of gaseous NH_3 that there was $[NH_3]g$ production at pH 8.8 but not at pH 6.6. At pH 8.8, gaseous NH_3 production rate relates to the concentration of $[NH_4^+/NH_3]aq$ in culture medium. A level of 1.5 mmol L^{-1} $[NH_3+NH_4^+]aq$ in the culture medium could inhibit Nase activity whatever the pH 6.8 or 8.8. NH_3/NH_4^+ produced by *A. variabilis* C9 could be consumed by itself at both pH 6.8 and pH 8.8 and Nase inhibited by high $[NH_3+NH_4^+]aq$ concentration could be reactivated when $[NH_4^+/NH_3]aq$ concentration decreased to an undectable level. Then, to find the condition always activating Nase activity and avoiding inhibited by the extracellular NH_4^+ could be furtherly investigated for continuous gaseous NH_3 production.

General conclusions and perspectives

Conclusions

The context of this study was the potential production of nitrogen bio-fertilizer from N_2 fixation by heterocytous cyanobacteria. The aim was to bring some knowledge on the feasibility of such a process. The species *Anabaena variabilis* and one of its MSX resistant strains, previously obtained by double random mutagenesis treatments, were cultivated in two sets of photobioreactors. These tools were used for improving the biomass and extracellular ammonium/ammonia productivities.

Some interesting features were highlighted in relation with the hypothesis we have deduced from a preliminary analysis of bibliographic data.

Under diazotrophic conditions, the biomass productivity up to 0.80 g L^{-1} d^{-1} was improved for the wild-type as well as the mutant strain. Moreover, it was shown that the mutant strain C9 has a better photosynthetic efficiency comparing to the wild type strain. The improvement was obtained through adapted programming the pH regulation of the photobioreactors, as under diazotrophy the pH variations are very negligible by contrast with cultures grown with nitrate or ammonium.

Another question was to measure the impact of the partial inhibition of the glutamine synthetase activity by MSX treatment on the biomass productivity. As well as in batch and in continuous cultures, the biomass production was not reduced. Moreover, continuous treatments with MSX at pH 6.8 resulted in a significant increase of steady state biomass productions by comparison with untreated cultures. At pH 8.8, known as a suboptimal pH value, the biomass production is partially inhibited, but in the same time relatively high concentrations of extracellular ammonium/ammonia were measured.

As already demonstrated by several authors, the MSX treatment in the range of the tested concentrations could result in a significant release of ammonium/ammonia. Our study has shown that the release of ammonia from cells has an impact on the pH of the culture. This original observation confirmed the hypothesis that dissociated form of ammonia seem to leak by diffusion through the membrane and the cell wall of heterocyts.

Another hypothesis deals with the impact of this pH variation on the distribution of ammonia and ammonium in the liquid culture medium. One of the expected results was that, as ammonia concentration is increasing, it could be transferred to the gas phase. Indeed, ammonia was detected in the gas phase and successfully quantified by trapping in an HCl solution. NH_3 liberation in batch culture occurred mainly at high pH (around 9.0) and maximal gaseous NH_3 productivity up to 1500 µmol g X^{-1} d^{-1} at an aeration rate of 2.4 vvm. Our results show that gaseous NH_3 production rates relate to the concentration of NH_4^+/NH_3 in the culture medium.

The effects of some environmental parameters on NH_3 production were characterized in batch cultures. We have confirmed that nitrogen fixation by heterocytous cyanobacteria is light dependent. In our culture conditions an optimal irradiance was 100 µmol m^{-2} s^{-1}. This value was obtained at a cell concentration 0.30 g L^{-1} dry cell weight in the mini-photobioreactors. This value could be different when using optimized cultures, as those obtained in the 0.7 L photobioreactors, with biomass up to 0.9 g L^{-1}. As the ammonia production is light dependent in heterocytous cyanobacteria, the self-shading process within highly concentrated cultures has to be taken in account. A global approach was to introduce the specific light supply rate (SLSR), for comparison. Relations between SLSR and biomass and combined nitrogen could be used in the future. Here it is interesting to note that maximal biomass productivity of the wild-type and mutant strains, at pH 6.8 with or without MSX, were obtained at SLSR values of about 0.6 mol photons g X^{-1} d^{-1}. By contrast, the maximal extracellular production of ammonium and ammonia productions were obtained at pH 8.8 with a SLSR of about 1.9 mol photons g X^{-1} d^{-1}. Such observations raise questions about the impact of the light transfer in photobioreactors on the physiological process involved in nitrogen fixation and ammonia release.

Our study has also highlighted that the NH_4^+/NH_3 productivity of this mutant strain is higher at 40 °C than at 30 °C. The cell viability was not decreased by such temperature. Moreover, the photosynthetic activity, as measured by the answer of the PSII from the vegetative cells, was significantly increased.

As hypothesized, the value of the aeration rate in the photo-bioreactor has a significant impact on the excretion of both NH_4^+ and NH_3 in the liquid phase (culture medium) but

mainly on the stripping of ammonia and its trapping in an acid solution. The k_La values of the setup used during the present study are relatively low. Then there seems to a room for improvement of the stripping through increasing the k_La. Changing the configuration of the air sparger for introducing smaller bubbles, then increasing the value of the k_La, within the cultures, is one of the classical ways to optimize gas/liquid transfer.

The preliminary results confirm that chemostat cultures with pH control are suitable to determine kinetics of biomass production, cell states and NH_4^+/NH_3 production.

Here, a cyclical production of extracellular ammonium/ammonia, we have obtained in batch cultures, was also obtained in chemostat cultures at pH 6.8 and pH 8.8 (**Figure 5−4** and **5−5**). It seems that a threshold level of 1.5 mmol L^{-1} NH_4^+/NH_3 in the culture above which the Nase activity seemed inhibited. Then, the decrease in the extracellular NH_4^+/NH_3 concentrations could be attributed to a cellular consumption, as there was no more gaseous ammonia stripped to the acid trap. As soon as the concentration of ammonium/ammonia in the liquid phase was under the limit of detection, the cyanobacterial population started restart to produce again extracellular combined nitrogen. The interpretation is that Nase activity is autoregulated by the variations of the combined nitrogen the cyanobacteria are producing or consuming in/from the culture medium. The chemostat cultures could be used for a further in-depth study of this cyclic production.

Some stoichiometric relations could be derived from data obtained during the present study. Indeed, the elemental composition of *A. variabilis* PCC 7937 was determined by L.A. Bui (2013) as $CH_{1.69}O_{0.487}N_{0.154}$ (Mx = 23.64 g mol^{-1}) close to the values $CH_{2.11}O_{0.485}N_{0.159}$ obtained by *Tsygankov et al. (1998)*. Then for biomass production (**Chap III**):

$$CO_2 + 0.845\ H_2O + 0.077\ N_2 \rightarrow CH_{1.69}O_{0.487}N_{0.154} + 1.179\ O_2$$

The stoichiometry shows that 0.077 moles of N_2 have to be fixed for producing one mole biomass, or 0.09 g N_2 per g of dry biomass. As the maximal biomass productivity for the wild-type strain was 800 mg L^{-1} d^{-1}. Then according to the stoichiometric equation, the nitrogen fixation can be estimated to 72 mg N_2 L^{-1} d^{-1}.

The elemental composition of the mutant strain *A. variabilis* PCC7937-C9, was determined by L.A. Bui (2013) as $CH_{1.725}\ O_{0.425}N_{0.2}$ (Mx = 23.33 g mol^{-1}).

When the GS activity is partially blocked, this stoichiometric equation could be written as:

$$CO_2 + aH_2O + bN_2 \rightarrow CH_{1.725}O_{0.425}N_{0.2} + c([NH_3]_{aq} + [NH_4^+]_{aq} + [NH_3]_g) + dO_2 + eOH^-$$

At first approximation, the extracellular ammonium/ammonia can be written "$[NH_3]_{tot} = [NH_3]_{aq} + [NH_4^+]_{aq} + [NH_3]_g$" for a nitrogen balance. Then, using the amounts of ammonia/ammonium and biomass produced during the batch cultures at high k_La value, where maximal accumulation was about 3.0 mmol L^{-1} of combined nitrogen (2.1 mmol L^{-1} $[NH_3 + NH_4^+]_{aq}$ and 0.9 mmol L^{-1} $[NH_3]_g$) from initial biomass concentration 0.3 g L^{-1} to the end (48h) biomass concentration 0.33 g L^{-1} (**Figure 4−10**):

$$CO_2 + 1.23\ H_2O + 0.23\ N_2 \rightarrow CH_{1.725}O_{0.425}N_{0.2} + 0.26\ "[NH_3]_{tot}" + 1.38\ O_2$$

According to such stoichiometry, an estimation of the amounts of N_2 fixed gives:

- 3.6 mg N_2 for 0.03 g L^{-1} biomass production
- 29.4 mg N_2 for the global production of 2100 µmol combined nitrogen, assimilated to [0.34 NH_3 + 0.66 NH_4^+] at pH 8.8 in the liquid phase
- 12.6 mg for the global production of 900 µmol combined nitrogen in the gas phase

These estimations highlight the fact, in these batch cultures:

- 7.9 % of the fixed nitrogen was assimilated for biomass production
- 64.5 % of the N_2 fixed was used to produce combined nitrogen in the liquid phase
- 27.6 % of N_2 fixed in these were stripped to the gas phase

For the continuous cultures, such estimations can be derived from data obtained at pH 8.8 and presented in **Table 5−1**. They correspond to the maximal concentrations of each combined form of nitrogen;

- 16.8 mg N_2 L^{-1} d^{-1} for 0.14 g L^{-1} d^{-1} biomass production
- 9.24 mg N_2 L^{-1} d^{-1} for the production of 660 µmol L^{-1} d^{-1} combined nitrogen, assimilated to [0.34 NH_3 + 0.66 NH_4^+] at pH 8.8 in the liquid phase

- 2.31 mg N_2 L^{-1} d^{-1} for the production of 165 µmol L^{-1} d^{-1} NH_3 in the gas phase

These estimations highlight the fact, in this continuous culture:

- 59.3 % of the fixed nitrogen was assimilated for biomass production
- 32.6 % of the N_2 fixed was used to produce combined nitrogen in the liquid phase
- 8.1 % of N_2 fixed in these were stripped from the culture medium.

This global nitrogen balance analysis highlights that the production of extracellular ammonia can be improved.

Perspectives

In this thesis, we have attempted to use a new strategy for gaseous NH_3 production from the mutant strain *A. variabilis* PCC 7937-C9 and investigated effects of several environmental parameters, especially the pH, on extracellular NH_3 production both in the batch and continuous cultures. Although progresses have been made, there are still large possibilities to improve and to continue this work in the future. Here are some suggestions for further studies.

1. Stopping of NH_4^+ uptaking process by *A. variabilis* to continuously produce gaseous NH_3

Cells start to uptake the NH_4^+/NH_3 in the culture after NH_4^+/NH_3 concentration reach the level of 1.5 mmol L^{-1} in the culture. This cell uptake process hindered continuous gaseous NH_3 production. Then, further investigation on how to stop cell uptake the NH_4^+/NH_3 from culture but continuously produce gaseous NH_3 could be meaningful. Further works could be done through strain selection or genetic transformations to control the NH_4^+/NH_3 concentration less than 1.5 mmol L^{-1} in the culture.

2. Gas balance between liquid phase and gas phase of the gaseous NH_3 production culture

Gaseous NH_3 production in *A. variabilis* culture is highly dependent on the aeration rate. Then, the second part work could be done to characterize the gas balance in the culture, such as how the N_2 ratio in the bubbling air and how the aeration rate affects the N_2 transfer

from gas phase into liquid phase and then fixed by cells for NH_3 production; and also, how the NH_3 transferred from liquid phase into gas phase.

3. New PBR design for mass culture of *A. variabilis* for bio–fertilizer production

Present studies only carried out in a 1 L small photo-bioreactor with good seal and stripping the gas phase from gas outlet. However, to strip the gas phase in mass culture for bio–fertilizer production could be a challenge. For this purpose, a special bioreactor could be designed with gas stripping and no release of NH_3 into atmosphere.

4. Investigation on the use of temperature for improving ammonia recovering

According to our results, the photosynthesis activities and NH_4^+/NH_3 productions are higher at 40°C than those at 30 °C. One aim could be to take advantage of this property. This temperature level could be relatively selective; more studies are needed for demonstrating that it could prevent the development of main microbial contaminants. Another interesting point is related to the fact that the ammonia water solubility is inversely related to temperature. Then further studies could focus on the effect of temperature variations, e.g. in the range 20 to 40°C, on the efficiency of liquid/gas transfer. Such relations could help for predicting gaseous ammonia production rate in solar photobioreactors, where the natural infrared radiations can be used for such purpose.

5. Gaseous NH_3 for supplying combined nitrogen to oleagineous microalgae

Considering the optimal gaseous NH_3 production occurs around pH 8.8, we can assume that about 79 µmoles NH_3 was daily stripped from the culture of *Anabaena* grown in the presence of 10 µmol L^{-1} MSX. Under the pressure and temperature, no detectable MSX is stripped through the gas phase. Then the gaseous ammonia can be trapped by a microalga culture in place of the acid solution used during the present study.

With our stoichiometric data, we can only estimate globally the volume of *Anabaena variabilis* necessary for the continuous production of a microalga; we have to know the N content of the microalga biomass and its volumetric productivity. For instance we could expect to get about 44 mg biomass L^{-1} d^{-1} *B. braunii* strain 30.81 cultures. On the basis of a maximal biomass productivity of *B. braunii* biomass of about 200 mg L^{-1} d^{-1}, a 5 L PBR of *Anabaena* would be necessary to provide combined nitrogen through gas stripping.

However, some feasibility studies are needed before the implementation of such two-stage-photobioreactors, to take in account the kinetics of the different physiological and physical processes involved for the valorization of gaseous ammoniac.

The model used for the present study was a mutant strain of *Anabaena variabilis* treated by MSX. Our results prompt for checking similar data could be obtained by using other cyanobacteria. Bibliographic data suggest that either some naturally occurring cyanobacteria, mainly symbiotic *Nostoc* species, or some genetically *engineered* species, e.g. with altered glutamine synthetase, could be used for next experiments. The behavior of these cyanobacteria could need a complementary study with the help of the continuous culture technology.

Nomenclature

A	light incident area (m^2)
Chl	chlorophylls concentration (mg L^{-1})
Chl *a*	chlorophyll a concentration (mg L^{-1})
Car	carotenoids concentrations (mg L^{-1})
D	dilution rate (d^{-1})
DIC	dissolved inorganic carbon (mg L^{-1})
ETR	electron transport rate (μmol electrons m^{-2} s^{-1})
ETR_{max}	maximal electron transport rate (μmol electrons m^{-2} s^{-1})
F	flow rate of feeding medium (L d^{-1})
Fg	gas flow rate (L min^{-1})
Fv/Fm	maximum photochemical quantum yield of PS II
k_La	volumetric gas mass transfer coefficient
PFD	photon flux density (μmol photons m^{-2} s^{-1} or μmol m^{-2} s^{-1})
Px	biomass productivity (g $L^{-1}d^{-1}$)
Px’	biomass productivity (g m^{-2} d^{-1})
SLSR	specific light supply rate (μmol photons $g^{-1}d^{-1}$)
T	temperature (°C)
V	volume (L)
X	biomass concentration (g L^{-1})

Greek letters

μ	specific growth rate (d^{-1})
μ_{max}	maximal specific growth rate (d^{-1})
λ	wavelength (nm)
τ	aeration rate (vvm)

Abbreviations

AcCoA	acetyl coenzyme A
ADP	adenosine double phosphate
APC	allophycocyanin

ARA	acetylene redution activity
Arg	arginine
As	asymbiotic strain
Asp	aspartate
Av (MHR) Edar	multiple herbicide ammonia excretory resistant strain of *A. variabilis*
A. circinalis	*Anabaena circinalis*
b/f	cytochrome b6f complex;
C-AGB	Ca–alginate gel beads
CYN	cylindrospermopsin
DCMU	3– (3,4–dichlorophenyl) –1,1–dimethylurea
EDA	ethylene–diamine
EMS	ethyl methanesulfonate
EPS	exopolysaccharides
F6P	fructose 6–phosphate
FL	free living
FNR	ferredoxin NADP reductase
GDH pathway	glutamate dehydrogenase pathway
Gln	glutamine
Glu	glutamate
GS	glutamine synthetase
GS-GOGAT pathway	glutamine synthetase–glutamate synthase pathway
Im	immobilized
M	mutant
MnSOD	manganese superoxide dismutase
MRP	mussorie rock phosphate
MSX	methionine–D, L–sulfoximine
Nase	nitrogenase
Nr	reactive nitrogen
OAA	oxaloacetate
PBPs	phycobiliproteins
PBR	photobioreactor
PBS	phycobilisome
PC	phycocyanin
PE	phycoerythrin

PetF	vegetative cell type ferredoxin
PGA	3–phosphoglycerate
Pi	inorganic phosphate
PSI	photosystem I
PSII	photosystem II
PUF	Polyurethane foam
PW	paper waste
R5P	ribose–5–phosphate
SCW	sugarcane waste
TCP	tricalcium phosphate
WPF	white polyvinyl foam
WT	wild type
2–OG	2–oxoglutarate
6PG	6–phosphogluconate

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List of publications and presentations

Papers for Journals

1. **Wenli KANG**, et al. Kinetic study of ammonia production by MSX treated *Anabaena variabilis* cultures with a continuous gas stripping [J]. 2016, Biotechnology and bioengineering (in writing).

2. Wenli KANG, et al. Biomass improvement and NH_3/NH_4^+ production by diazotrophic continuous cultures of *Anabaena variabilis* with pH regulation [J]. 2016, Algal research (in writing).

Presentations at congress

1. Dominique GRIZEAU, **Wenli KANG**, Jian JIN, Catherine DUPRE. Procédés adaptés à l'intensification de productions d'exométabolites par des microalgues et cyanobactéries; contraintes et perspectives. Présentation au Congrès International de Biotechnologie et de Valorisation des Bio–Ressources, Mars 24 –27, 2016, Hammamet (Tunisie).

2. **Wenli KANG**, Catherine DUPRE, Lan Anh BUI, Jack LEGRAND, Dominique GRIZEAU. Continuous cultures for production of biomass and extracellular combined nitrogen by a diazotrophic cyanobacteria mutant. Oral presentation at international conference "ECCE10+ECAD3+EPIC5", September 27–October 1, 2015, Nice (France).

3. **Wenli KANG**, Lan Anh BUI, Jian JIN, Catherine DUPRE, Jack LEGRAND, Dominique GRIZEAU. Physiological inverstigations of diazotrophic cyanobacteria cultivated in chemostat photobioreactors: improvement of biomass productivity. Poster presentation at 7th Asia–Pacific Biotech congress, July 13 –15, 2015, Beijing (China).

4. Catherine DUPRE, Astrid De LUCA, Olivia CLAUDE-LACHENAL, **Wenli KANG**, Jian JIN, Jérémy PRUVOST, Jack LEGRAND, Dominique GRIZEAU. A medium–throughput screening (MTS) strategy for microalgae and cyanobacteria characterization. Poster presentation at 7th Asia–Pacific Biotech congress, July 13 –15, 2015, Beijing (China).

Curriculum vitae

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Research Interest

Cultivation of cells in bioreactor for production of high value products

UNIVERSITE BRETAGNE LOIRE

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Wenli KANG

Kinetic study of ammonium/ammonia production by *Anabaena variabilis* cultures in relation with a continuous gas stripping

Etude des cinétiques de production d'ammonium/ammoniaque dans des cultures de *Anabaena variabilis* en relation avec un stripping continu par la phase gazeuse

Résumé

Certaines cyanobactéries photoautotrophes sont capables de fixer l'azote atmosphérique grâce à des cellules spécialisées, les hétérocytes. De plus, en aérobiose, comme ces cellules peuvent excréter de l'ammonium lorsque leurs activités glutamine synthétase sont partiellement inhibées. Elles sont considérées comme usines cellulaires potentielles pour une bioproduction d'engrais azoté. Nous utilisons une souche mutante de Anabaena variabilis PCC 7937-C9, cyanobactérie hétérocytée à taux de croissance élevé, pour étudier la capacité à produire de l'ammonium en photobioréacteurs. Les caractéristiques de croissance de cette souche ne différent pas significativement de celles de la souche sauvage, avec un taux de croissance spécifique maximal de 3.0 j^{-1} à 30°C. Nous montrons qu'une partie de l'azote excrété dans le milieu de culture est entrainé sous forme de NH3 par la phase gazeuse, expliquant ainsi des sous-estimations antérieures. Cette production dépend de la température, l'irradiance, le taux d'aération et la concentration en MSX. Des études cinétiques confirment que la production d'azote ammoniacal en phase liquide et en phase gazeuse est corrélée aux variations de pH. Une régulation pulsée de pH permet d'accroitre la production de NH_3. Des cultures en chemostat confirment que les productions de NH_3 gazeux sont maximales à pH 8.8. Une variation cyclique des teneurs en NH_4^+/NH_3 dissous semble réguler les teneurs en NH_4^+/NH_3 en dessous d'un seuil critique de 1.5 mmol L^{-1} via une consommation par les cellules végétatives. Ces caractéristiques physiologiques sont analysées pour une application potentielle à la fourniture d'azote à des cultures de microalgues oléagineuses.

Mots clés
cyanobactéries, *Anabaena variabilis*, souche mutante, ammoniaque, L-methionine sulfoximine, photosynthèse chemostat, stripping gazeux

Abstract

Some photoautotrophic cyanobacteria species are able to fix dinitrogen thanks to specialized cells, the heterocyts. Moreover, these cells are known to secrete ammonia when the glutamine synthase activity is partially inhibited under aerobic conditions. They are considered as potential cell factories for fertilizer. The present study uses a mutant strain of Anabaena variabilis PCC 7937-C9, a fast-growing heterocytous cyanobacterium, to investigate the potential use of diazotrophic cyanobacteria in photobioreactors for ammonium production. The growth characteristics of this strain cultivated in chemostat cultures are not significantly different from those of the wild strain, with a maximal specific growth rate of 3.0 d^{-1} at 30°C. A part of the combined nitrogen excreted in the culture medium is shown to be stripped through the aeration of the cultures as NH_3, indicating previous underestimation of NH_4^+/NH_3 excretion. This process is shown to be affected by parameters such as temperature, irradiance, gas flow rate and MSX concentrations. Kinetics study reveals that the dissolved NH_4^+/NH_3 as well as the gaseous NH_3 productions are correlated to pH variations production; a pulse regulation of pH is used to increase the NH_3 production. Chemostat cultures with pH regulation are used to confirm that maximal gaseous NH3 is produced at pH 8.8. A cyclic variation of dissolved NH_4^+/NH_3 seems to regulate the NH_4^+/NH_3 concentrations under a threshold level of 1.5 mmol L^{-1}; uptake of NH_4^+ by vegetative cells seems to be involved. These physiological features are discussed in view of operative conditions for efficient nitrogen supply for production by oleaginous microalgae.

Key Words
cyanobacteria, *Anabaena variabilis*, mutant strain, ammonia, L-methionine sulfoximine, photosynthesis, chemostat; gas stripping