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Effects of N source and nitrification pretreatment on growth of *Arthrospira platensis* in human urine*

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Abstract: Culture of *Arthrospira platensis* (*Spirulina platensis*) in human urine was investigated to get valuable biomass. NO_3-N was the proper N source, in comparison with other N source, including urea, NH_4-N and NO_2-N. As a result, aerobic nitrification of human urine was performed, with above 93.6% nitrification percentage finally achieved with total-N (TN) load of 46.52 mg/(L·d), in which *Arthrospira platensis* was successfully grown. The main compositions of the obtained biomass are close to those in Zarrouk medium. Thus, it is possible to culture *Arthrospira platensis* in nitrified human urine for food production within bioregenerative life support systems (BLSSs).

Key words: *Arthrospira platensis*, Human urine, N source, Nitrification, Bioregenerative life support system (BLSS)
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INTRODUCTION

To support long-term human stay in space, bioregenerative life support systems (BLSSs), including higher plants, aquatic organisms and unicellular microalgae are being designed and tested to fully regenerate atmosphere, water and partially food (Gòdia *et al*., 2002; Wheeler *et al*., 2003; Blüm *et al*., 2003).

Microalgae were extensively incorporated in BLSS (Tadros *et al*., 1991; Salisbury and Gitelson, 1997; Gòdia *et al*., 2002). Compared with higher plants and aquatic organisms, they are completely edible and their cultivation process is faster, simpler and highly reliable (Salisbury and Gitelson, 1997). Of them, *Arthrospira platensis* (*Spirulina platensis*), which now generally recognized as a kind of cyanobacterium (Soletto *et al*., 2005), was applied because of its rich nutrition (Belay, 2002; Tadros *et al*., 1991) and has been applied as food in Mexico and Chad for nearly 500 years (Richmond, 1992). Animal tests proved *Arthrospira platensis* is safe as food or food additive (Chamorro, 1980). Moreover, its composition can be controlled to satisfy the crew's taste by altering the culture condition, culture medium components and their concentration within BLSS (Tadros *et al*., 1991).

Human urine is one of the main sanitary wastes produced by crew member, and contains only 5% of organic matters, but has 50% to 90% of nitrogen, phosphorus and potassium (Larsen and Gujer, 1996). Furthermore, it contains all necessary trace elements for culture of *Arthrospira platensis* (Gòdia *et al*., 2002), which includes B, Cu, Zn, Mo, Fe, Co and Mn (Rodushkin and Ödman, 2001). Thus, human urine shows potential to be applied for the culture of photoautotrophic *Arthrospira platensis*.

In our preliminary experiments, *Arthrospira platensis* was grown successfully in untreated diluted human urine (Feng and Wu, 2006). Nevertheless, the obtained biomass is small in size, easy to precipitate and deficient in protein content. Thus, effects of N source on culture of *Arthrospira platensis* in human

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urine were investigated. As a result, pretreatment of diluted human urine by nitrification was proposed, and culture of *Arthrospira platensis* was subsequently examined. Our purpose was to get valuable biomass in pretreated human urine, which may then be introduced into BLSS for food production.

MATERIALS AND METHODS

Arthrospira platensis and culture medium

Arthrospira platensis 439 was obtained from the Freshwater Algae Bank of the Chinese Academy of Sciences (CAS). The culture medium used in this experiment contained Zarrouk medium (ZM) (Gòdia *et al*., 2002), real human urine (RHU) and synthetic human urine (SHU) (Gordon, 1982). ZM (1000 ml) is composed of 18.0 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1.0 g K_2SO_4, 1.0 g NaCl, 0.04 g $CaCl_2$, 0.08 g Na_2EDTA, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, and 1.0 ml trace elements (TEs). TEs (g/L): H_3BO_3 2.86; $(NH_4)_6Mo_7O_{24}$ 0.02; $MnCl_2{\cdot}4H_2O$ 1.8; Cu_2SO_4 0.08; $ZnSO_4{\cdot}7H_2O$ 0.22, with culture medium pH being adjusted to 8.2 by 1 mol/L NaOH solution. SHU (1000 ml) composed of 0.5 g $CaCl_2{\cdot}2H_2O$, 4.12 g K_2HPO_4, 0.47 g $MgCl_2{\cdot}H_2O$, 0.29 g KCl, 4.83 g NaCl, 1.55 g NH_4Cl, 2.37 g Na_2SO_4, 13.34 g urea, 1.0 g creatinine and 0.65 g sodium citrate, with its pH being 6.8, which is close to RHU (pH 6.5~7.5). SHU and RHU are 10-times diluted prior to use, unless otherwise specially mentioned. Main compositions of ZM and SHU are the same as previously described in short communication (Feng and Wu, 2006).

Bubble column photobioreactor

The bubble column photobioreactor (Fig.1) designed and used in our experiments was 60 cm high, 7.2 cm in diameter and 1200 ml in culture volume. A concentric fluorescent lamp in an enclosed glass tube submerged in the culture medium was set to provide light energy to *Arthrospira platensis*. Light and dark cycle was controlled to 14:10 by a microcomputer timer switch. At its bottom, a circular air bubble generator was placed to provide CO_2 for the photosynthesis of *Arthrospira platensis*. The generated air bubble can agitate the culture medium to avoid the accumulation of O_2 and self-shading of *Arthrospira platensis*. The culture medium temperature within the photobioreactor can be adjusted and kept constant by water bath. At the top of the photobioreactor, a sampling port and a gas outlet were set for sampling and gas discharge respectively. Seven uniform photobioreactors were used to do the experiments.

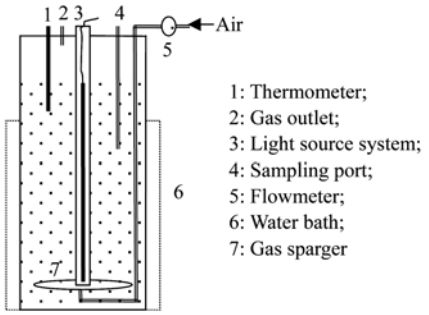

Fig.1 Schematic diagram of bubble column photobioreactor

Culture of *Arthrospira platensis*

Arthrospira platensis in its log phase in ZM was concentrated by filtration, and inoculated to culture medium to initiate the culture. Culture parameters applied in this experiment were as follows: temperature: 30 °C, light energy provided per unit surface area: 444.4 W/m^2 and aeration flow rate: 3 L/min. During culture, deionized water was added to culture medium daily to compensate water loss by evaporation.

Nitrification of human urine

A packed-bed reactor with 5.6 L culture volume was used in this experiment. Porous ceramic ring was chosen as nitrifying bacteria carriers, which are about 12 mm long and have external and inner diameter of 14 and 8 mm respectively. The volumetric surface available for bacterial growth was assumed to be 206 m^2/m^3. The filling volume percentage of ceramic rings to the total volume (7.6 L) was about 26.3%.

During the whole nitrification process, temperature was kept at 27 °C. Aeration of air (6 L/min) was done to keep the aerobic condition within the bioreactor. pH was adjusted to 8 daily by addition of 125 g/L Na_2CO_3 solution. When starting the nitrification process, nitrifying culture medium (Gòdia *et al*., 2002) was firstly used to accumulate nitrifying bacteria and form biofilm on the ceramic ring surface. Subsequently, diluted SHU and RHU were added into the bioreactor to be nitrified.

Analytical methods

pH of culture medium was measured by METTLER TOLEDO 320s pH meter (Mettler-Toledo Instruments (Shanghai) Co., Ltd.); Algae biomass concentration was measured daily by optical density (OD_{560}) and was plotted versus dry weight (DW, g/L) on a standard curve (Costa *et al*., 2004). Water, protein, lipid, total chlorophyll, carotenoid, dissolved oxygen (DO) concentration, ash, TN, NO_2-N, NO_3-N and NH_4-N were analyzed according to the standard methods (APHA, AWWA, WEF, 1995; Sullivan and Carpenter, 1993). Metallic elements contents of *Arthrospira platensis*, including K, Na, Ca, Mg and Fe were determined using inductively coupled plasma-atomic emission spectrometry (ICP-AES, Thermo Iris Intrepid II XSP). Linear growth rate (v, g/(L·d)) (Ogbonna *et al*., 1995) was used to evaluate the growth rate of *Arthrospira platensis*, with its value being calculated according to the equation:

$$v=(DW_t-DW_i)/t, \quad (1)$$

where v is the linear growth rate, t is the culture time, DW_i and DW_t is the biomass concentration at the beginning and end of the linear growth phase respectively. Each growth curve was calculated after logistic curve fitting, the maximum value of the curve is then defined as the maximum productivity (P_{max}, g/L), which was used to evaluate the final biomass concentration.

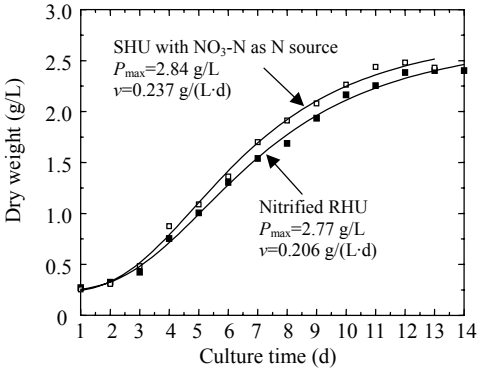

Fig.2 Batch culture results of *Arthrospira platensis* in SHU with NO_3-N as N source and in nitrified RHU

RESULTS AND DISCUSSION

Effects of N source

Arthrospira platensis was first cultured in SHU with NO_3-N ($NaNO_3$) as N source in batch mode, to obtain v of 0.237 g/(L·d) and P_{max} of 2.84 g/L (Fig.2), in comparison with that cultured in ZM with v of 0.342 g/(L·d) and P_{max} of 3.74 g/L. The obtained biomass was green, cannot easily settle, with its chlorophyll (Chl) A, B and C contents showing little difference from those of ZM (Fig.3). Furthermore, *Arthrospira platensis* in NO_3-N group was rich in protein (54.6%) (Table 1), and the contents of water, lipid and ash were also on the same level as those in ZM.

Effects of NO_2-N were also tested (data not shown). NO_2-N may also accumulate in human urine under low DO, high NH_3 concentration, extreme pH, and high temperature condition (Udert *et al*., 2003; Ruiz *et al*., 2003). *Arthrospira platensis* cannot survive in SHU with NO_2-N as the sole N source. In order to eliminate its prohibition effects, NO_2-N

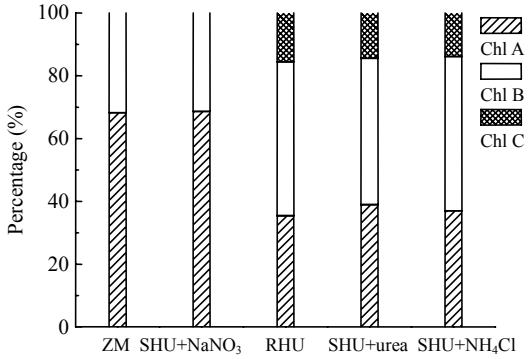

Fig.3 Chl A, B and C contents of *Arthrospira platensis* cultured in ZM, 180 times diluted RHU and SHU with urea, NH_4Cl and NO_3-N as N source. Dilution ratio of SHU with urea, NH_4Cl and NO_3-N as N source were 180, 180 and 10 respectively

Table 1 Main compositions of *Arthrospira platensis* cultured in SHU with 90% NO_3-N+10% NH_4Cl (A) and 100% NO_3-N (B) as N source

N source	Composition (per 100 g DW)					
	Water (g)	Protein (g)	Lipid (g)	Total Chl (mg)	Carotenoid (mg)	Ash (g)
A	4.83	53.61	10.86	1417.56	237.84	7.51
B	5.31	54.60	9.53	1328.62	244.48	8.68

concentration within diluted SHU should be kept below 26.5 mg/L.

Effects of urea and NH_4-N were finally examined. In order to avoid prohibition of high NH_4-N concentration on *Arthrospira platensis* and N deficiency within diluted SHU, intermittent addition of urea and NH_4-N was applied here to keep a low but stable NH_4-N concentration within the culture medium (Soletto *et al*., 2005). Feeding protocol (Rangel-Yagui *et al.*, 2004) of urea and NH_4Cl in this experiment followed the equation:

$$m_i=m_0+bt^c, \quad (2)$$

where m_i is the added amount of urea or NH_4Cl (mmol/(L·d)), m_0 is the starting value of m_i, b and c are feeding parameters. After carefully tested, m_0, b and c of urea group were set to be 1.0 mmol/(L·d), 0.15 mmol/(L·d^2) and 0.5 respectively, and those of NH_4Cl group were 2.0 mmol/(L·d), 0.3 mmol/(L·d^2) and 0.5 respectively to keep the same NH_4-N addition level. During the culture, NH_4-N accumulation occurred only when entering into the stationary phase, but maximal NH_4-N concentration of urea group (2.65 mg/L at day 16) and NH_4Cl group (6.20 mg/L at day 14) (Fig.4) were still far below the inhibitory threshold (47.6 mg/L) (Soletto *et al*., 2005), which showed a successful feeding protocol. Low NH_4-N accumulation for urea group was due to the limited urea hydrolyzing rate, although it may be accelerated under alkaline conditions (Carvajal *et al*., 1980).

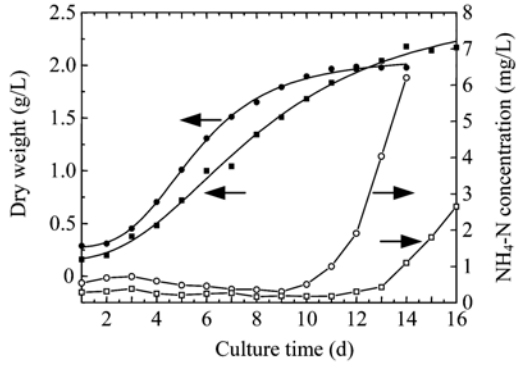

Fig.4 Biomass growth (solid) and NH_3-N accumulation (hollow) during fed batch culture of *Arthrospira platensis* in 180-diluted SHU with urea (rectangle) and NH_4Cl (circle) as N source. Intermittent addition of different N sources was performed according to Eq.(2) with urea of m_0=1.0 mmol/(L·d), b=0.15 mmol/(L·d^2), c=0.5, and NH_4Cl of m_0=2.0 mmol/(L·d), b=0.3 mmol/(L·d^2), c=0.5

The culture results of *Arthrospira platensis* in synthetic human urine with urea and NH_4-N as N source are also shown in Fig.4. Although intermittent addition of urea and NH_4Cl was done, their biomasses were still yellow-green, small in size and easy to precipitate. Moreover, their Chl A, B and C contents were sharply different from those of ZM, but close to those in diluted RHU (Fig.3). All these culture results indicated that urea and NH_4Cl are not the proper N source as they lead to the increase of Chl C and B. Using urea and NH_4-N as N source done by Danesi *et al*.(2002), Rangel-Yagui *et al*.(2004) and Soletto *et al*.(2005) also got satisfactory results. In our observation, NO_3-N seems to be the proper N source for culture of *Arthrospira platensis* in human urine.

Fig.5 shows the effect of NO_3-N level on Chl A, B and C contents of *Arthrospira platensis* cultured with NH_4Cl as the other N source used to balance and keep a total-N concentration of 0.663 g/L (equivalent to total-N concentration in SHU). Fig.5 shows that when NO_3-N accounted for over 90% of the N source, Chls contents show little variation. However, when it fell from 90% to 85%, Chls contents drastically varied, in which Chl A decreased from 65.46% to 49.42%, Chl B increased from 32.76% to 40.38% and Chl C increased from 0 to 10.20%. As a result, the main composition of *Arthrospira platensis* cultured in SHU with 90% NO_3-N as N source was analyzed. The results showed little difference from that with 100% NO_3-N as N source (Table 1), which is in accordance with the variation in Chl A, B and C contents. Thus, in order to get high quality of *Arthrospira platensis*, proper treatment, such as nitrification, should be

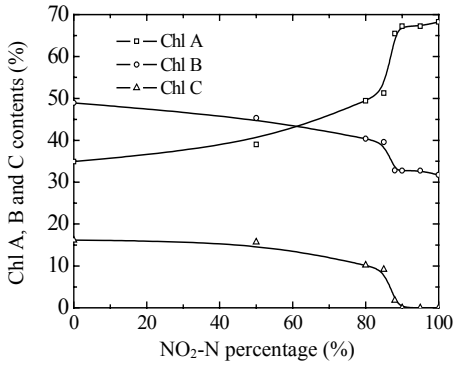

Fig.5 Chl A, B and C contents of *Arthrospira platensis* cultured in SHU with different NO_3-N levels. NH_4Cl was compensated into the SHU to get equivalent total-N concentration to that in SHU (0.663 g/L)

made to convert no less than 90% NH_4-N in RHU to NO_3-N, and meanwhile to keep NO_2-N concentration less than 26.5 mg/L.

Nitrification of RHU

After nitrifying bacteria accumulated within the bioreactor, nitrification of 8-diltued SHU with TN load of 88.77 and 59.18 mg/(L·d) was done, but no more than 62.5% nitrification percentage was obtained (data not shown). Subsequently, TN load was further reduced to 50.00 mg/(L·d) by increasing the dilution ratio of SHU to 10, with the RHU nitrification results being shown in Fig.6. From day 1 to 12, an increasing in nitrification percentage was achieved gradually, and stabilized within the next 4 d, with the value above 94.95%. From day 16 to 34, the influent was substituted by RHU, and no less than 93.6% nitrification percentage in this stage was successfully achieved. The effluent from day 28~34 was collected and stored under 4 °C to be used to culture *Arthrospira platensis*.

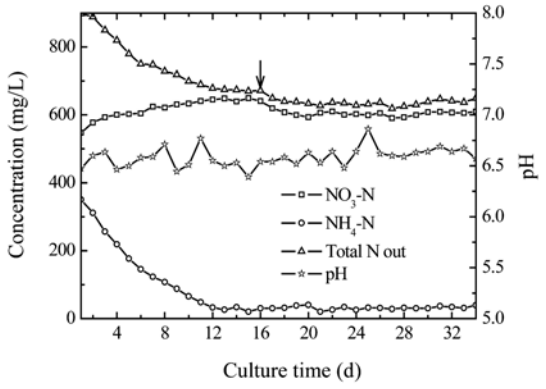

Fig.6 Nitrification results of SHU and RHU. From day 1 to 15, SHU was added as culture medium with influent rate, influent TN concentration and TN load of 400 ml/d, 699.95 mg/L and 50.00 mg/(L·d) respectively. From day 16 ("↓") to 34, RHU was added into bioreactor with influent rate, influent TN concentration and TN load of 400 ml/d, 651.29 mg/L and 46.52 mg/(L·d) respectively

For nitrification of wastewater with high NH_4-N concentration (such as human urine), decreasing in amount, community and growth rate of ammonia oxidizing bacteria has been observed (Alenka *et al*., 1998; Mota *et al*., 2005). Moreover, during nitrification process, N loss by evaporation of NH_3 would occur, which is negatively correlated with NH_4-N concentration. Thus, in order to avoid the inhibition of nitrification by high NH_4-N concentration, and to minimize N loss, human urine was diluted before nitrification. A nitrification strategy with decreasing N concentration and load gradually was performed, and verified successfully. N loss decreased from about 6.97% to the finally 2.42% (Table 2), which is a really low level. Yet, this process was focused on converting NH_4-N in human urine into NO_3-N within BLSS. Thus, further treatment should be taken to avoid N loss.

Table 2 N loss throughout the nitrification process

Culture time (d)	TN concentration (mg/L)	TN load (mg/(L·d))	N loss±*SD* (%)*
7th~13rd	1749.88	355.06	6.97±2.34
18th~24th	1749.88	177.53	6.26±2.17
30th~36th	1166.59	118.35	5.08±1.52
41st~47th	874.94	62.50	4.89±1.19
53rd~59th	699.95	50.00	3.76±1.34
64th~70th	651.29	46.52	2.42±1.72

* six days average

Accumulation of the unexpected NO_2-N may also occur concomitantly (Ruiz *et al*., 2003). Moreover, the formed NO_2-N and NO_3-N may also denitrify to N_2, NO and N_2O (Ahn, 2006), which will result in N loss. O_2 concentration has proved to be the most important factor inhibiting accumulation of NO_2-N, and the reduction of NO_2-N and NO_3-N (Ahn, 2006; Colliver and Stephenson, 2000), Thus, DO within the bioreactor was stabilized between range 4.23~5.14 mg O_2/L to keep an aerobic condition within the bioreactor. Throughout the nitrification process, no NO_2-N was detected, and high NO_3-N percentage (94.95%) and low N loss (2.84%) showed that high DO inhibited the accumulation of NO_2-N and denitrification process successfully.

pH within the bioreactor stabilized within the range of 6.54 and 6.92 throughout the culture process. Also in the nitrification experiment (data not shown) without pH control, pH decreased sharply to 5.83 and stabilized on this level, while only about 50% nitrification percentage was achieved. Udert *et al.*(2003) also obtained similar results. Stein and Arp (1998) attributed these phenomena to the lack of NH_3, which is said to be the true substrate of ammonia oxidizing bacteria (Kowalchuk and Stephen, 2001), while the real reason may need further investigation.

Batch culture results of *Arthrospira platensis* in nitrified RHU are typically shown in Fig.2. *Arthrospira platensis* grew successfully with P_{max} of 2.77 g/L and v of 0.206 g/(L·d), which showed little difference from that of SHU with NO_3-N as N source. The obtained biomass was green, not easy to deposit and showed size similar to that in ZM. In addition, a proportion of its main composition (Table 3), including protein (58.43%), lipid (10.67%), ash (7.64%) and total chlorophylls (1376.97 mg/100 g DW) was close to that of ZM, which were 67.20%, 11.93%, 6.5% and 1328.62 mg/100 g DW, respectively. For metallic elements contents of *Arthrospira platensis* cultured in nitrified RHU, except Mg (1897.48 mg/100 g DW) contents was a little higher than that in ZM (337.679 mg/100 g DW), K, Ca, Na and Fe contents differed little from those in ZM. The culture results indicated that nitrification of RHU is a feasible way to improve the growth rate and quality of *Arthrospira platensis*.

Table 3 Main composition of *Arthrospira platensis* cultured in nitrified RHU

Composition (per 100 g DW)	Value
Water (g)	5.17
Protein (g)	58.43
Lipid (g)	10.67
Total Chl (mg)	1376.97
Carotenoid (mg)	264.37
Ash (g)	7.64
K (mg)	708.64
Na (mg)	806.95
Ca (mg)	211.87
Mg (mg)	1897.48
Fe (mg)	43.34

CONCLUSION

Among urea, NH_4Cl, NO_2-N and NO_3-N, NO_3-N ($NaNO_3$) seem to be the best N source for culture of *Arthrospira platensis*. Aerobic nitrification of human urine into NO_3-N is a proper way to get high quality biomass of *Arthrospira platensis*.

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