Agricultural Journal 2 (6): 672-675, 2007
ISSN: 1816-9155
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Growth Conditions of Associative Nitrogen-Fixing Bacteria *Enterobacter cloace* in Rice Plants

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Abstract: J115 and G161 are two strains of endophtic bacteria *Enterobacter cloacae* with strong associative nitrogen-fixing ability were isolated from rice stems and roots, respectively. The growth conditions of these bacteria were studied. The optimum time of culture for these bacteria was about 36 h and growth temperature was ranged from 27-30°C. Similarly, the optimum pH value of media was ranged from 6-7. The results showed that aeration or vibration culture (120 r min^{-1}) was beneficial to the bacterial growth.

Key words: Associative nitrogen-fixing bacteria, *Enterobacter cloace*, growth conditions, rice

INTRODUCTION

In the 21st century, one of the emergent problems facing human society is the production of enough foods to meet the needs of increasing population. Moreover, in order to reduce the pollution, protect the environment and save energy and resources, bio-fertilizers are more important than chemical fertilizers (Monzote, 2006). Among all sorts of chemical fertilizers, nitrogen fertilizer is the most important nutrient resource that is indispensable to crop growth. Generally, nitrogen in the air can not be directly used by most plants. However, nitrogen fixation bacteria can transform nitrogen element into ammonium nitrogen that used by plants, which is called bio-nitrogen fixation. Bio-nitrogen fixation from bacteria has two types, the symbiotic nitrogen fixation in legume and the associative nitrogen fixation in soil (Perotti, 1926). Nowadays, the interests of scientists in the world are focus on bio-nitrogen fixation research from bio-chemistry and molecular point of view. The endophyte is bacteria colonizing plants (De Bary, 1866), in 1870 Pasteur started research on the bacteria colonizing in the tissue of asymptomatic plants.

Kleopper *et al*. (1992) thought that the endophytes were those microorganisms which colonize in or between the cells of plant tissues and organs harmoniously. Nitrogen fixation bacteria can enter into rice plants by cranny caused by the development of lateral roots and root epidermal cells (Qianli *et al*., 1999). The nitrogen-fixing bacteria *Beijerinckia fluminensis* from tropic sugarcane was first isolated in 1958 by Dobereiner (1961) he found the associative nitrogen-fixing bacteria *Azospoirillum* in the rhizosphere soil of Poaceae in 1975 and then proposed the definition of rhizosphere associative nitrogen-fixing bacteria. The *Enterobacter bloacae* is a facultative anaerobic bacterium Gram negative (G^-) with straightly bacillar shape and round end, peri-flagella; utilizing sugar as carbon source; producing lactic acid and H_2, CO_2 and NH_3 during cultivation; fixing nitrogen on or in rice plant (Petrini, 1991; Wilson, 1995). About 21 species of nitrogen-fixing bacteria were isolated in the rhizosphere of rice plants in Pakistan, the prominent populations are *Pseudomonas* and Flavobacterium (Malik *et al*., 1997).

MATERIALS AND METHODS

Bacterial Isolation: Both G161 and J115 strains of *Enterobacter cloacae* were endophytic bacteria with high nitrogen fixation; they were isolated from stems and roots of rice plants in the laboratory of Plant Pathology at Yangzhou University, China.

Bacterial concentration standard-curve (cfu mL^{-1}) vs absorbance: Small piece of the bacterial culture was placed into the peptone broth, cultured with slight shake (80r min^{-1}) at 30°C. The absorbance of the bacterial culture was measured by spectrophotometer UV755B at 625nm. One milliliter of bacteria was transferred 3 times into 9 mL broth to obtain a serial dilution and then 0.2 mL of bacteria in each dilution was placed on the BPA plate evenly. The plate was incubated at 30°C for 72 h to record the number of bacterial colonies. The standard curve was given the relationship between the logarithm of the bacterial concentration (cfu mL^{-1}) and the Absorbance (A).

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Effect of cultivation time on bacteria growth: One milliliter of bacteria culture which was cultivated at 30°C for 22 h was transferred into 200 mL sterile broth culture. The broth was shake at 30°C/120 rpm and the absorbance of the broth at each cultivation time 12, 24, 36, 48, 60, 72, 84, 96 and 108 h was measured by spectrophotometer UV 755B at 625 nm.

Effect of cultivation temperature on bacteria growth: One milliliter of bacteria culture which has been cultivated at 30°C for 22h was transferred into 50 mL sterile broth. The solution was shaking at 24, 27, 30, 33, 36 and 39°C under 120 rpm for 30 h and its absorbance was measured at 625 nm.

Effect of pH on bacteria growth: The sterile broths with labeled pH 4, 5, 6, 7, 8, 9, 10 and 11 were adjusted by NaOH. Thereafter, 1 mL of bacteria culture which has been cultivated at 30°C for 30h was transferred, its absorbance was measured by spectrophotometer UV 755B at 625 nm.

Effect of shaking condition on bacteria growth: One milliliter of bacteria culture which has been cultivated at 30°C for 22h was transferred into 150 mL sterile broth shaked fewer than 120 rpm. Cultivate samples for 48 h under quiescent condition were shacked 12h interval. The absorbance was determined at 625nm by spectrophotometer UV 755B. For all experiments the absorbance of sterile broth was used as blank control and then the number of bacterium in different condition was calculated.

RESULTS

Standard curve of bacteria concentration (lgcfu mL^{-1}) vs absorbance: The relationship equation between strain J115 concentration and its absorbance was Y = 7.9775X + 0.0135 (Fig. 1). While, the relationship equation between strain G161 concentration and its absorbance was Y =8.0392X-0.1477 (Fig. 2).

Effect of culture time on the bacterial growth: After 12h culture the number of G161 and J115 strain of nitrogen-fixing bacteria were rapidly increased. Thereafter, the bacteria were slowly increased relatively to the time of culture. The number of the bacteria cultured reached to the maximum at 36h and then was persist at a stationary stage (Fig. 3).

Effect of culture temperature on the bacterial growth: The growth of strain J115 and strain G161 reached the maximum at 27-30°C, respectively (Fig. 4).

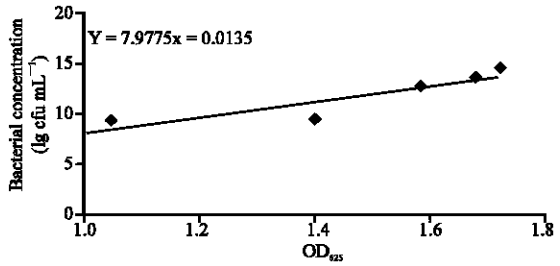

Fig. 1: Relationship between the absorbance at OD_{625} and the concentration of strain J115

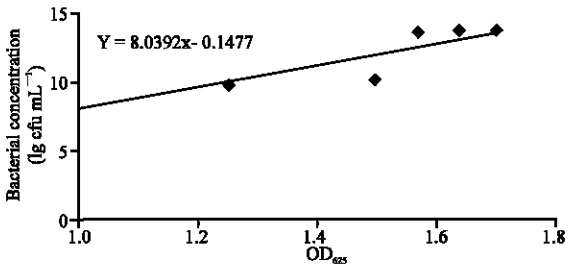

Fig. 2: Relationship between the absorbance at OD_{625} and the concentration of strain G161

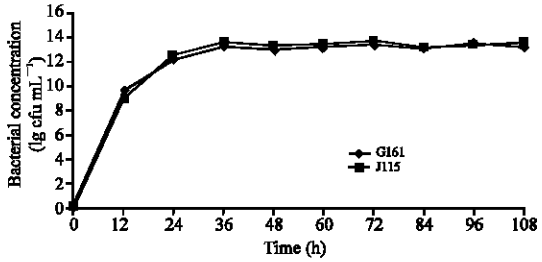

Fig. 3: The growth of the bacteria in different culture time

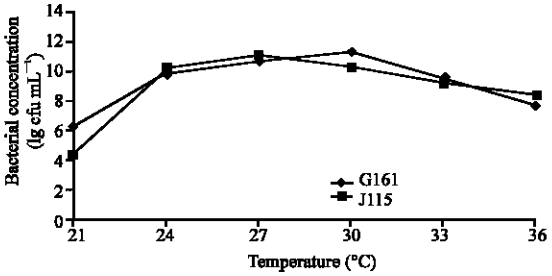

Fig. 4: The growth of the bacteria at different temperature

Effect of media pH on the bacterial growth: Figure 5 showed that both two bacteria could not grow when the pH value of the media was 4. The growth of bacteria increased rapidly when the pH of media was increased and the number of bacteria reached the maximum at pH

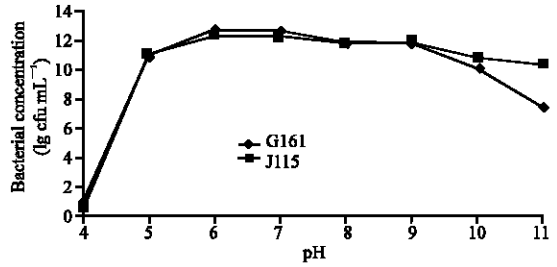

Fig. 5: The growth of bacteria in the media with different pH value

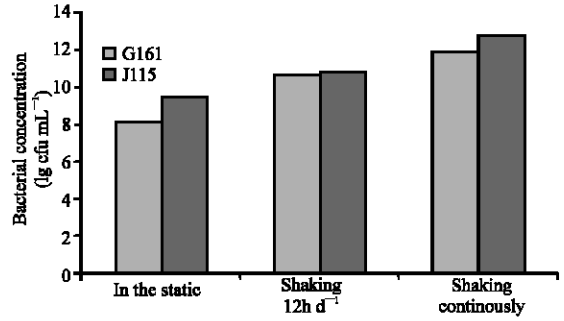

Fig. 6: The growth of the bacteria under different shake conditions

6~7. Thereafter, the growth of bacteria decreased with the increased of pH. It suggested that the optimal media pH for the growth of two nitrogen-fixing bacteria was ranged from 6-7.

Effect of vibration culture on the bacterial growth: Although two bacteria were facultative anaerobes, were grew better under proper vibration than in the static, the best vibration for bacteria growth was (120 rpm) (Fig. 6).

DISCUSSION

The results of growth condition of both bacteria strains showed that, the optimal condition to get the maximum number of bacteria were 36-48h, 27-30C and 6-8 pH. The aerobic condition is good for the nitrogen fixation bacteria growth. According to the economical factors, the optimal components for the growth of nitrogen fixation bacteria were 2% glucose, 1% peptone, 0.02% K_2HPO_4 and 2% yeast extract for strain J115. While for strain G161 were 2% glucose, 2% peptone, 0.02% K_2HPO_4 and 1% yeast extract (Dobereiner *et al*., 1993). Although, the concentration of the two bacterium strains when cultured in the media containing $(NH_4)_2SO_4$ were not reduce, it is not consistent to the conclusion that the ammonium ceasing phenomena is not exist. To prove whether, the two strains have the same phenomenon, the growth and nitrogen fixation of the bacteria will cost a lot of energy for the fixation of nitrogen effectively. However, high quantity of nitrogen fertilizer will inhibit the synthesis and the activity of nitrogen fixation (Sturz *et al*., 2000). Research on the recombinant nitrogen fixation bacteria is very important. In Japan researchers colored the nitrogen fixation gene *nif* into the associative nitrogen fixation bacterium firstly and construct the gene engineering bacterium, which have good nitrogen fixation ability under the high concentration of NH_4 (Van Peer *et al*.,1996). The researchers were constructed and screened many recombinant nitrogen fixation bacteria with ammonium resistance. For example, by cloning the gene *ntrC* and *nifA*, a new recombinant gene engineering bacteria *Al. faecalis* were constructed and the nitrogen fixation ratio can reach at 20. More attention is posting to the endogenesis nitrogen fixation bacteria these days and most countries and researchers are devoting research on endogenesis nitrogen fixation bacteria. We believe that the endogenesis nitrogen fixation bacteria will be applied in the agricultural production broadly. It will also bring active effects and will bring favorable economy benefit.

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