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Studying biological characteristics and classification of bacterium capable of cellulose biosynthesis isolated from wastewater after biogas plants

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Abstract: Wastewater treatment is always an urgent issue. Especially, the wastewater after the biogas plants does not have adequate attention. In wastewater treatment studies, the use of biological measures is the first priority. From the wastewater samples after the biogas plants, 3 bacterial strains isolated which is highly capable of cellulase biosynthesis identified as NT01, NT03 and NT05. The paper presents the biological, morphological, physiological and biochemical characteristics of 3 strains are all thermophilic, suitable pH 5 ÷ 8. They are capable lysis of CMC, starch and dilution of gelatin. Cellulase biosynthesis of NT01 strain reaches 2.28 IU/ml, NT03 strain reaches 2.14 IU/ml and NT05 strain reaches 2.12 IU/ml. Identification of the behavior of the strains in which the optimal growth time is 42 hours, the growth of the NT01 strain reaches 2,155, the strain of NT03 reaches 2.89, the NT05 strain reaches 2.715. Use of 50CHB Kit, molecular biology technique 16S rARN to do species identification: the NT01 species is B. *licheniformis* NT01, the NT03 strain is B. *subtilis* NT03 and the NT05 strain is B. *megaterium* NT05.

Keywords: *Wastewater, treatment, biogas, cellulase, behavior*

I. INTRODUCTION

Currently, agricultural wastewater in Vietnam is mainly treated with anaerobic digestion, after this process the components that pollute environment are still very high. The treatment of wastewater of biogas plants before discharging into the environment is very necessary and need to treat simultaneously many agents, especially organic substance, nitrogen and phosphorus. There are many technical solutions that are deployed for research and application, depending on the scale, including biological methods. The outstanding advantage of microbial technology in treating environmental pollution is reducing costs compared to other methods. Simultaneously, this technology is also very efficient and saves energy, which is a favorable condition to treat them with biotechnology to meet human life. For the treatment process of wastewater, the environmental conditions such as salinity, acidity, alkalinity, components of organic substances, ... greatly affect the growth and decomposition efficiency of organic substances. This paper will present the results of the study on biological characteristics and classification of some bacterium that are capable of biosynthesis of cellulase isolated from wastewater after the biogas plants.

II. MATERIAL AND METHOD

2.1 Material

Microbial strains: Selected microorganism strains are NT01 strain, NT03 strain and NT05 strain. The strains are kept in the breed collection of the Department of Biological Materials Technology, Institute of Biotechnology, Vietnam Academy of Science and Technology

Materials: Biochemical Standard Kit 50 API CHB. Primer couple for bacterial classification:

Pr16F: AGAGTTTGATCCTGGCTCAG.

Pr16R: TACGGTTACCTTGTTACCGACTT

Culture medium;

- MPA culture medium: meat glue 3; pepton 5; NaCl $_5$; agar 20; water 1000 ml.
- Agar cellulase culture medium (g/l):NH_4NO_3 0,2; ure 01; cazein 0,2; KH_2PO_4 0,2; $MgSO_4.7H_2O$ 0,3; $CaCO_3$ 0,2; $FeSO_4.7H_2O$ 5mg, pulp 20; yeast glue 0,1; Cazamino axit 0,1; agar 20.

2.2 Methods of study

Methods of studying microorganisms: Evaluation method of the antagonism between bacterial strains [2]. Study method of factors affecting growth [2]: temperature, pH, NaCl concentration. Method determined activity of cellulase. Method reduced sugar following Bernfeld [3].

Classification of bacteria according to API 50 CHB standard kit: bacteria strain is cultured by 24-hour in the MPA medium, adding 10% of culture fluid into API 50 CHB medium, shaking well. Sucking API 50 CHB has the microorganism strain, then, injecting into the chips, after that, dripping about 5 ÷ 6 drops of paraffin into the mouth of chips to prevent the infection and evaporation of the fluid. Raising at 37 0C after 24 ÷ 48 hours, recording results. Sorting by molecular biology based on the sequence of 16S rARN genes:

a) Extraction of bacteria genomic DNA: The extraction process of bacteria genomic DNA was conducted according to CTAB/NaCl method of Ausubel et al., 1994.
b) Cloning DNA fragment of 16S rARN gene by PCR: Obtaining 16S RNA gene fragment by PCR reaction.
c) Determination of the sequence of 16s rRNA gene: DNA sequence is determined by the Dyedeoxy Terminator Cycle Sequencing kit, the product analyzed on the ABI 377 automatic sequential reader machine [1].

III. RESULTS

The strains are determined the lysis capacity of substrates are CMC and starch, using culture method by points on the plate, incubated at 37 ^{0}C for 48 hours.

3.1 Biological characteristics of the bacterial strains studied

Table 1. The lysis capacity of CMC and starch of the bacterial strains

Order	General symbols	Lysis round (D-d (± 2mm))	
		CMC	Starch
1	NT01	21,0	20,0
2	NT03	19,0	17,0
3	NT05	17,0	15,0

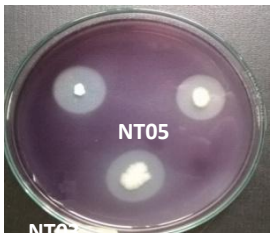


Figure 1. The lysis capacity of CMC

Figure 1 and Table 1 show that these strains are high capable of cellulase biosynthesis, in accordance with the research objectives. The bacterial strains are culture in the liquid MPA medium with addition of 10% gelatin, cultured at 37 0C. The fluid of the medium is dilute, meaning that the strains is capable of diluting gelatin.

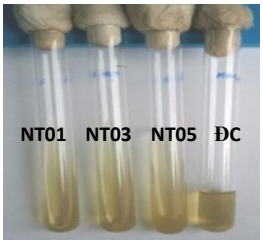


Figure 2. Capacity of diluting gelatin of the bacterial strains

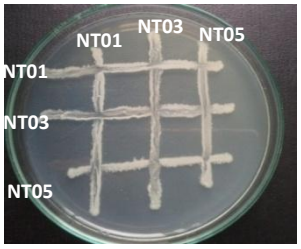


Figure 3. Capacity of the antagonism between the bacterial strains

The results in Figure 2 show that the bacterial strains are able to dilute gelatin better than the control sample without microorganism and in Figure 3 shows that the strains are not antagonistic. All three bacteria grow normally at intersections, with no inhibition between the strains. Thus, they can fully grow in the same environment as well as combine them in organic wastewater treatment.

3.2 Morphology characteristics of colony and cell

Observing colony morphology and cell images taken at the Department of morphology, Institute of 69, the Ho Chi Minh Mausoleum high command.

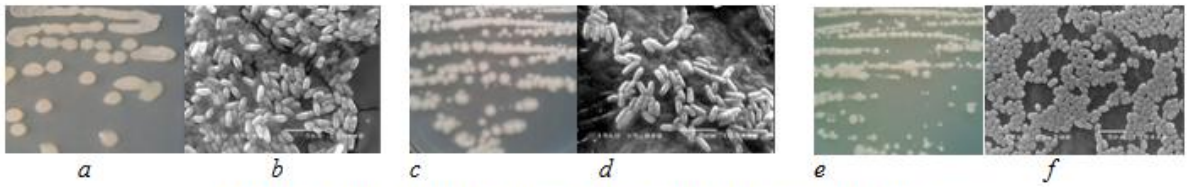
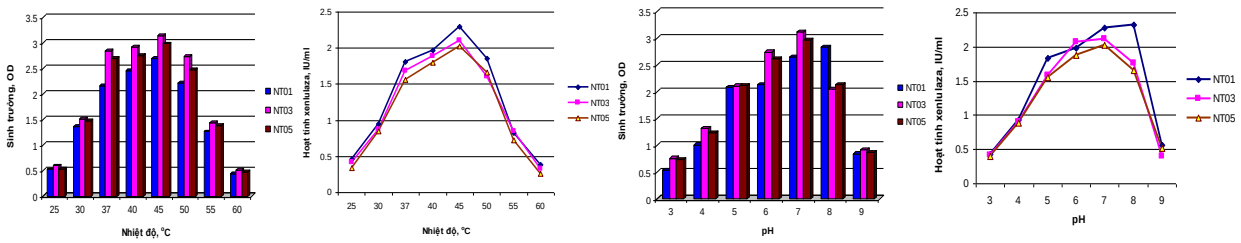


Figure4. Morphology of colony and cells of the bacterial strains
a; b. NT01 strain; c; d NT03 strain; e; f. NT05 strain

Figure 4 shows two bacterial strains of NT01 and NT03 all have long rod-shaped cell, size from 1 ÷ 3 µm. The cell morphology of the two strains is similar but the colony morphology is completely different, the colony of the NT01 strain is convex, non-glossy, mucous, slightly wrinkled, the colony of the NT03 strain is milky white, slightly convex, serrated edge. The NT05 strain has shorter rod-shaped cells, size 1 ÷ 2 µm, the colony morphology of NT05 is round and creamy white.

3.3 Effect of temperature, pH on the ability of growth and biosynthesis of cellulase of the bacterium

Resistance ability of high temperature is an important criterion in treatment applications. We cultured bacterial strains in the temperature range: 25, 30, 37, 40, 45, 50, 55 and 60 ^{0}C. With the objective of selecting the strain that is capable of cellulase lysis for treating wastewater, the microorganism has ability of surviving in a wide range of pH is essential. The bacterial strains were cultured in the liquid MPA medium, temperature at 37 ^{0}C, shake 200 rounds/minute, and the pH range of the medium is: 3 ÷ 9.



a/ b/ c/ d/

Figure 5. Effects on growth and cellulase biosynthesis of bacterial strains
a; b/ Temperature effect;c; d/ pH effect

The results show that the strains are able to withstand relatively high temperatures, at 25 ÷ 30 ^{0}C and greater than 50 ^{0}C, the growth is weak but from 37 ÷ 50 ^{0}C the growth of the strains (OD) is very good, all 3 strains optimally grow at 45 ^{0}C, the growth capacity of the NT01 strain is 3.76, the NT03 strain is 3.82 and the NT05 strain is 3.8. Normally, at the temperature of the best growth bacteria, the production of cellulase is highest. The cellulase biosynthesis temperature of three strains is from 37 ÷ 50^{0}C and optimum temperature is at 45 ^{0}C, the activation of the NT01 strain reaches 2.3 IU/ml, the NT03 strain reaches 2.1 IU/ml and NT05 reaches 2.0 IU/ml. If the culture temperature is less than 37^{0}C higher than 50^{0}C, the cellulase production ability of all the strains decreases. The results of the above study show that all three strains belong to the warm-loving microorganisms group, they are suitable for requirements of wastewater treatment. Strains selected can grow in a wide range of pH from 3 ÷ 9, optimal pH of the NT01 strain is 8, growth ability (OD) reaches 3.95, the NT03 strain reaches 3.81, the NT05 strain reaches 3.78 and optimal pH is 7, when the pH value <5 and ≥ 9, the growth of the strains decrease. With pH 7 is optimal pH for cellulase biosynthesis of 2 strains NT03 (reaching 2.1 IU/ml) and NT05 (reaching 2 IU/ml), the NT01 strain has the highest ability of cellulase biosynthesis is at pH 8 reaching 2.28 IU/ml.

3.4 Growth behavior of the bacterial strains

Study on the growth behavior of the bacterial strains shows that the optimal time for growth of 3 strains is 42 hours, OD of the NT01 strain reaches 2.155, NT03 reaches 2.89, NT05 reached 2.715. From 6 ÷ 42 hours the strains grow very fast. From 42 hours onwards, the growth of strains decreases. The quantity of cellulase produced increases continuously but relatively different between 3 strains, in Figure 6 shows that the NT03 and NT05 strains are very large biomass produced but the capacity of cellulase biosynthesis is not as high as that of the NT01 strain. The other two strains are also achieved the highest cellulase quantity after 48 hours, the NT03 strain reaches 2.5 IU/ml, the NT05 strain reaches 2.45 IU/ml. After 48 hours the quantity of cellulases produced by all 3 strains decreases.

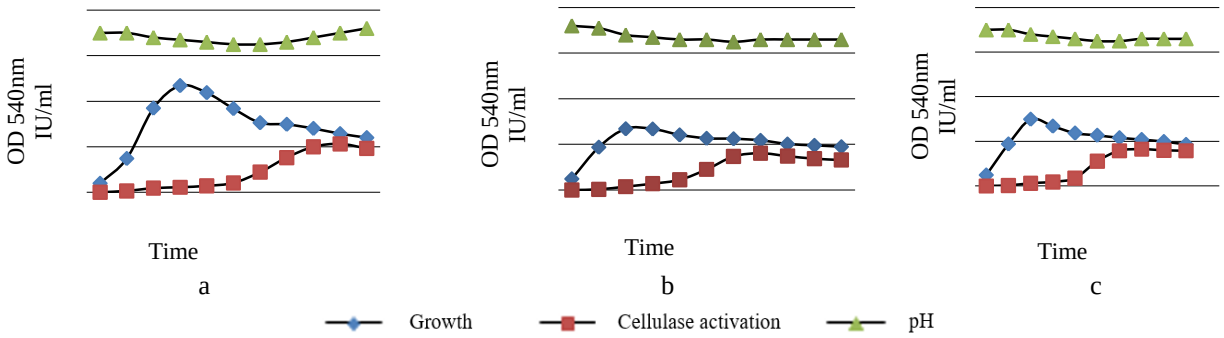


Figure 6: Growth behavior of 3 bacterial strains
6a. NT01 strain; 6b. NT03 strain; 6c. NT05 strain

3.5 Classification characteristics of the bacterial strains studied

Usability of API 50 CHB Biochemical Standard Kit: API 50 CHB kit uses classification of Gram (+) bacteria belonging to Bacillus strains that are relatively specific, classification characteristics are done based on usability of 49 carbon sources.

Figure 7. Photos using the 50 CHB Kit of the bacterial strains
a/ NT01 strain; b/ NT03 strain; c/ NT05 strain

Comparing with API profile index, combining with morphological characteristics according to Bergey's bacteriological classification, showing that the determination level of the NT01 bacterial strain belongs to the Bacillus licheniformis species (% ID = 91.4 and T = 0 , 88), denoted by B. licheniformis NT01, the NT03 bacterial strain belongs to the Bacillus subtilis species (% ID = 88.5 and T = 0.96), denoted by B. subtilis NT03 and the NT05 strain belongs to the Bacillus megaterium species (% ID = 84.6 and T = 0.76), denoted by B. megaterium NT05.

3.6 Classification by molecular biology

The electrophoresis result in Figure 8 shows that PCR product obtained a very specific band. Molecular size is about 1,500 bp is equivalent to theoretical calculation.

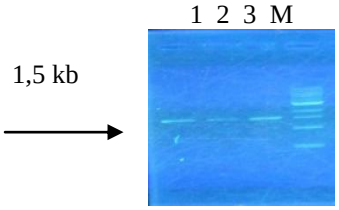


Figure 8. PCR product electrophoresis of the bacterial strains
Notes of figure 8:
1: NT01; 2: NT03; 3: NT05; 4: Marker

3.7 Sequence of the 16S rARN gene of 3 bacterial strains studied

PCR product after purification used directly to determine the nucleotide sequence of the 16S rARN gene. Using fluorescent-mounted primer fraction of the chemical standard set, analyzation is done by automatic DNA sequence determination machine, processed by PC/GENE program. To access the gene bankfinding the registered species which have homologous sequences.

Table 2. The homologous level of the 16S gene of the bacterial strains with the sequence of strains in the GenBank

Order	Symbol	16S rRNA gene sequence	Homology	Homologous with the author
1	NT01	CAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAG CGGCGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGA CTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTT GTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCT ACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGA GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC GAAAGTCTGACGGAGCAACGCCGCG	100 % of homology compared to*theBacillus licheniformis species*	Lianyungang, Jiangsu, China 2011
2	NT03	TGCGCAAGCTTAGAGTTTGATCCTGGCTCAGGACGAACGCT GGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGG GAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAAC ACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA AACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTT CAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGA CCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC AAGGCAACGATGCGTAGCCGA	100 % of homology compared to *the Bacillus subtilis species*	Oyetibo,Glori, G. Endo and M.F. Chien, 2012
3	NT05	GGCCAGTGCGGCGTGCCTATACTGGCAAGTCGAGCGAAC TGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGGACGGG TGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATA ACTTCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTC CTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACT TACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGT AACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGA GGGTGATCGGCCACACTGGGA	100% of homology compared to the *Bacillusmegaterium species*	S.Akbar and M.Kertesz 2013

The results in Table 2 show that the 16S rRNA sequence of the bacterial strains is analyzed by Chromas program. Using Clustalw program to compare sequences of the bacterial strains with the GenBank data bank. The results shows that it is possible to classify the NT01 bacterial strain as B. *licheniformis*, the NT03 bacterial strain as B. *subtilis* and the NT05 bacterial strain as B. *megaterium*.

IV. CONCLUSION

Studied on morphological, physiological and biochemical characteristics of 3 bacterial strains of NT01, NT03 and NT05 are Gram (+) strain, thermophilic, resistance ability of NaCl from 1 ÷ 5%, suitable pH from 5 ÷ 8 , capable of lysis of CMC, starch as well as diluting gelatin. Used Bergey's classification key in combination with API CHB Biochemical Standard Kit, together with 16S molecular biology technique to classify to species, the NT01 bacterial strain is B. *licheniformis* NT01, the NT03 bacterial strain is B. *subtilis* NT03 and the NT05 bacterial strain is B. *megaterium* NT05.

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