MICROBIOLOGYCAL CHARACTERIZATION AND POTENTIAL APPLICATION OF INDIGENOUS *B.METHYLOTROPHYCUS* Ba_1 IN HANDLING OF *CANNA EDULIS.* KER PROCESSING CRAFT VILLAGE WASTEWATER

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SUMMARY

Canna edulis. Ker starch processing wastewater from craft villages contains high organic concentration such as starch, cellulose, xylan, protein… and especially high starch concentration after the states of grinding, sedimentation and filtration. The concentration of COD and BOD_5, SS and coliform of wastewater are hundreds times higher than standards. To handle and recover organic pollutants of this wastewater, we focus on strains that are able to efficiently convert organic pollutants into biomass. The microbial biomass along with activated sludge – a kind of material can be applied to many other purposes are removed out by settling well while processing. From starch processing sludge and wastewater, strain of *Bacillus methylotrophycus* Ba_1 was selected with notable features: having the ability to use a variety of substrates (starch, cellulose, xylan, protein) with highest amylase activity (D/d = 12.5), COD removed efficiency (90% after 12 hours) and good sedimentation of activated sludge with SVI = 120.4. The results also showed that the optimal conditions for its biomass production: temperature 30^{o}C, initial pH 6.0 and inoculum of 7% (v/v). After 24 hours incubation at these conditions, the highest biomass was measured OD_{600} of 2.91.

Keywords: Activated sludge, aerobic, Bacillus methylotrophicus, selection, wastewater treatment.

I. INTRODUCTION

Currently, the world's wastewater treatment technologies are working toward the combination of treating and recycling. In this opinion, waste is considered avaiable and recoverable resources - waste to energy (W2E). To handle and recover organic pollutants, microbial technology is the most popular, friendly, safe and sustainable application. Utilization of aerobic microorganism to process and metabolize pollutants into valuable product is one of new technological applications which are being received broad and enthusiastic concerns by scientists in the world.

Many reports have pointed out the effectiveness of using microbial for starch processing wastewater treatment. When studying on the growth of *Aspegillus oryzae* IFO 30113 in the cassava starch processing (CSP) wastewater, Truong Quy Tung *et al* showed that strain *A.oryzae* IFO 30113 was satisfiable not only for the production of fungal biomass but also for high efficiency wastewater treatment. After 96-hours incubation, high biomass yield (up to 0.8 g/g-COD) was achived and 87% total organic carbon (TOC), 91% COD and 94% starch were removed. When using combination strains of *candida utilis, A. niger* and *B. pumilus* to treat and convert potato processing wastewater into single cell protein (SCP), Bingnan Liu *et al* also showed that the COD of the wastewater was reduced from 26.700 mg/l to 9.100 mg/l and the SCP products, with a crude protein content of 46.09% (higher than soybean meal), were found palatable and safe for mice.

In Vietnam, The possibility for treating of organic effluents by using microorganisms has been studied previously. However, the knowlege is limited about combination of treating and utilizing of SPW to make valuable products like biomass, microbial fertilizer and others.

The aim of this study is to select and characterize bacterial strains having ability to reduce the pollution resulting from C*anna edulis*. Ker starch processing by transferring the organic pollutants contained in wastewater into biomass. Selected strains can be u

sed as a microbial consortium for bio-treatment and bio-conversion of craft village wastewater.

II. MATERIALS AND METHODS

2.1. Materials

2.1.1. Samples collection

Samples were collected in pre-sterilized polypropylene bottles from *Canna edulis*. Ker starch processing village, Minh Hong-Bavi, Hanoi, stored at 4˚C for isolation and screening of microorganisms according to TCVN 4556-88.

2.1.2. Chemicals and quipments

Chemiclas: PGA, Tris HCl, EDTA, SDS, protease K, β-mecaptoethanol, CTAB, chloroform, isoamyl alcohol, isopropanol, etanol, TE, dNTP, $MgCl_2$, PCR buffer, Taq polymerase, agarose), EtBr, BSA, K_2HPO_4, $(NH_4)_2SO_4$; KCl, $MgSO_4$, $FeSO_4$.$7H_2O$, $NaNO_3$, glucose, yeast extract, peptone, skim milk, meat extract, CMC, xylan, agar, starch, lugol... were made by manufacturers: Merck; Sigma and Wako in Japan, USA, Germany and China.

NA medium (10g/l peptone, 5g/l NaCl, 3g/l meat extract, 18g/l agar) was used for isolating.

Medium for determing the possibility of using substrates (MD): NA medium supplied with substrates: starch, CMC, skim milk and xylan at concentration of 1% for each.

Initial pH of media was set at 7.0 then the media was autocalved at 121^{o}C for 20 mins.

Equipments: sterilization autoclave HIRAYAMA (Japan), pH meter, biological safety cacbinet, Thermostat cabinets SHELLAB, rotator shaker VBEG - ML02, PCR equipment, electrophoresis quipment,...

2.2. Methods

2.2.1. Wastewater characteristics determination

Periodically and conventional parameters such as pH, TSS, Temp, COD and BOD_5 were determined as per the procedure recommended by QCVN 40:2011/BTNMT.

2.2.2. Isolation and screening methods

Samples were heated at 80^{o}C for 20 mins then serially diluted in distilled water and subsequently plated onto NA plates, incubated at 35^{o}C for 24 hours and cololies which distinct morpholygy were picked up and purified by regular subculturing.

Bacterial isolates were initial screened base on their abilities to use a variety of organic compounds (starch, cellulose, protein, xylan) contained in wastewater. These abilities were assessed through the secretion of respective extracellular enzymes. The formation of clear zone in the MD surrounding the wells or colonies indicated positive enzyme activity.

Next screening was carried out base on their ability to remove COD. Each selected strain was cultivated for 24h in nutrient broth (NA medium without agar). A 5% (v/v) of the inoculum was then transferred into 3 liter tants containing 2 liters of wastewater at a concentration of COD 2000 mg/l, after a period of 24 hours and/or up to 72 h at room temperature, continuous air supply then the percentage of COD moving was estimated. Control was performed without introducing bacteria. Strains that exhibited a high potential of COD moving were chosen for further experiments.

Final screening was carried out base on their biomass production and their activated sludge settling potential. 5% (v/v) inoculum of activated sludge from selected strains was supplied into tank containing 3 liters of wastewater, continuous air supply after a period of 72 h. Using 100ml cylinder and oven

to determine SVI (sludge volume index) of activated sludge, according to Luong Duc Pham, 2012.

2.2.3. Genotypic characterization method

The 16S rRNA gene sequencing of selected strains were carried out according to Sakiyama et al (2009). DNA extraction was performed as describing of Neunann et al (1992). The PCR reaction was conducted by Perkin Elmer 9700 machines with the primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR products after purifying was tested by 1% agar electrophoresis and sequenced as described previously (Tanasupawat *et al*., 2004). The nucleotide sequence of selected strains was compared with the sequence of 16S rRNA regions in GenBank database by means of BLAST search.

2.2.4. Effects of culture conditions on the growth of selected strains

Effect of temperature: Experiments were performed at (20^oC, 25^oC, 30^oC, 35^oC, 40^oC, and 45^oC), shaking at 150 rpm. The initial pH of incubation medium was set to 7.0 and culture time was 24 hours.

Effect of initial pH and inoculum rate: The ranges of pH set (3, 4, 5, 6, 7, 8 and 9), inoculum rate was introduced at the ranges (1%, 3%, 5%, 7%, and 10% v/v), shaking at 150 rpm, 30^oC.

Fermentation broth was used to estimated biomass through OD_{600} measuring on spectrometers.

III. RESULTS AND DISCUSSION

3.1. Characterization of *Canna edulis.* Ker processing wastewater

Canna edulis. Ker processing wastewater collected from craft village: Minh Hong – Bavi, Hanoi was brown and black in color with sour smell. it was observed that variations in pH of untreated effluent ranged from 4.8 - 5.2 and COD: 4154 - 5560 mg/l, BOD_5: 2357 - 2958 mg/l, SS in effluent were 649 - 815 mg/l, total Phosphor 45.76 - 49.72 and total Nitrogen 197.53-221.34, coliform $3.6*10^4$ – $4.5*10^5$ CFU/ml. The organic pollutants of the effluent which had adverse effects on aquatic flora, fauna and human beings, was found to be much higher than the permissible limit. Further, the rate of $BOD_5/COD \geq 0.5$ shows that this wastewater source is entirely impossible to be treated by microorganisms.

3.2. Bacterial isolation and Screening

To isolate bacterial strains having ability to treat *Canna edulis*. Ker wastewater, at first we selected strains having potential to efficiently use and convert wide range of organic compounds, especially in wastewater like starch, cellulose, protein and xylan.

Several bacterial colonies of distinct morphology and color were observed on nutrient agar plates after incubation at 35^oC for 24 hours. Twenty strains were isolated base on their enzyme activities. Among them, 4 strains named Ba_1, C_5, N_4 and T_2 were identified as aerobic, positive gram, capable of generating diverse enzymes, especially amylase activity of Ba_1 strain. The results show in table 1.

Table 1. Halo diameter produced by 4 strains (after 24 hours)

Strain	Cell shape, Gram	Catalase	Colony morphology on NA agar media	Halo to colony diameter ratio (D/d_1) subtracting well from halo diameter (D/d_2)			
				Starch (D/d_1)	CMC (D/d_1)	Skim milk ($D-d_2$)	Xylan ($D-d_2$)
Ba_1	Rod, alone or double, $G^{(+)}$, spores	+	Round, creamy white, convex surface, jagged edges, adhesive agar	12.5	5.1	12.5	10.2
T_2	Rod, square head, alone, $G^{(+)}$, spores	+	Round,flat surface, rough, serrated edges, adhesive agar	2.1	8.4	3.6	5.3

Strain	Cell shape, Gram	Catalase	Colony morphology on NA agar media	Halo to colony diameter ratio (D/d_1) subtracting well from halo diameter (D/d_2)			
				Starch (D/d_1)	CMC (D/d_1)	Skim milk ($D-d_2$)	Xylan ($D-d_2$)
C_5	Rod, $G^{(+)}$, spores	+	Milk white, smooth edges, heart flowers	2.3	7.1	4.7	0.8
N_4	Rod, $G^{(+)}$, Spores	+	Flower shape, yellow, jagged edges, rough	1.5	7.2	5.5	2.5

D: Halo diameter (mm); d_1: Colony diameter, d_2: well diameter

Table 1 shows 4 strains: Ba_1, C_5, N_4 and T_2 have ability to use wide range of substrates. Especially, Ba_1 strain has capable of releasing diverse enzyme and strong activity of amylase (D/d = 12.5), that is much higher than NT1 strains (D/d = 5.0) isolated previously. To test their application potential, the ability to remove COD of wastewater was determined.

Figure1 shows that 4 strains Ba_1, C_5, N_4 and T_2 were clearly expressed ability to remove COD, much higer than control. Among them, strain Ba_1 showed the fastest adaptability and the most effective metabolism of COD. After 12 hours processing, strain Ba_1 removed about 90% COD while strain N_4 removed 85%, T_2 : 75%, C_5 : 82% and control just 50% after 18 hours. Therefore, two strains Ba_1 and N_4 were selected for futher studies.

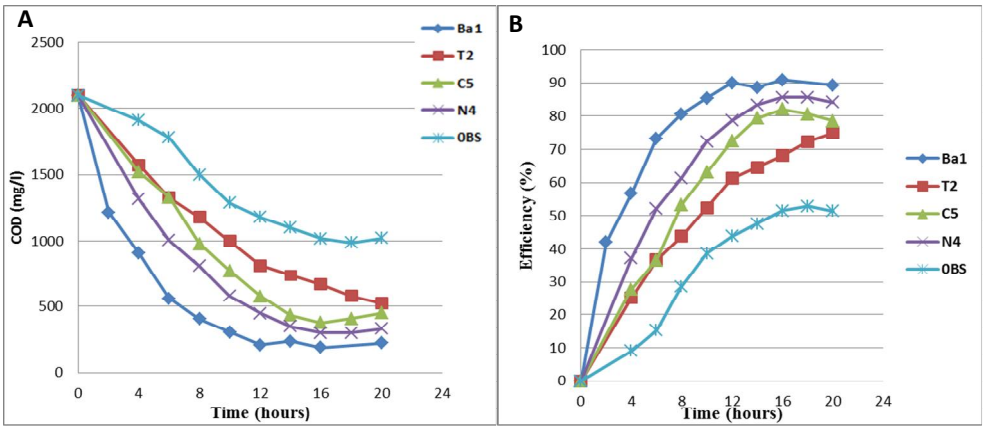

Figure 1. COD values (A) and COD removal efficiency (B) of wastewater

In any wastewater biological treatment processes, flocculation and separation is one of the most important steps. Therefore, beside ability to use diverse organic substrates and efficiently remove of COD, strains need to settle well. Two strains Ba_1 and N_4 were tested for their activated sludge settleability. The results in table 2 indicate that activated sludge from Ba_1 strain settled fast, good flocculation with SVI value measured 120.4, water after seedling was clear. Strain N_4 settled slower and not thorough.

Table 2. SVI values and characteristic of activated sludge from Ba1 and N4 strains

Strain	V total (ml)	V_{30} (ml)	MLSS (g)	M(g)	SVI	Sludge properties
Ba_1	1000	122	11.83	8.3	120.4	Brown, good flocculation, rapid settling
N_4	1000	105	7.56	5.22	191.57	Black, slow sedimentation

Many authors recognised SVI as the parameter best characterising sludge settling properties. In practice, SVI can vary from 30 to 400 ml/g. However, it usually does not exceed the value of 150 ml/g which is an indicator of good settling properties of the sludge. Palm and Jenkins reported that sludge of the SVI over 150 ml/l is often classified as bulking sludge. The same authors also have found out that quickly settling sludge (SVI below 70 ml/g) can be the reason for turbid effluent, caused by weakly structured and small floes.

3.3. Identification of selected strain

For molecular identification, genomic DNA was extracted from the isolated bacterial strain Ba_1. After sequencing, the nucleotide sequence of Ba_1 was compared with the sequences of 16S rRNA regions in GenBank database by means of BLAST search. Results showed that the 16S rRNA sequence of Ba_1 strain was highly homologous to *Bacillus methylotrophycus, Bacillus amyloquefacien, Bacillus vallismortis, Bacillus subtilis* that were widely applied in many different industries.

On the basis of 16S rRNA gene sequence, Ba_1 was closely related to *Bacillus methylotrophicus*_EU194897 and *Bacillus amyloliquefaciens* _NR041455 (1398/1400 bp) with 99.86% sequence similarity.

As shown in figure 2, the phylogenetic tree indicated that strain Ba_1 identified as *B. methylotrophycus*. The isolate Ba_1 has high biological safety thus will be useful to improve the potential applications in wastewater treatment.

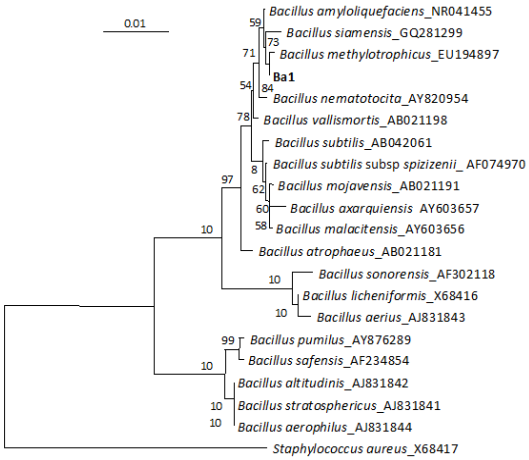

Figure 2. Phylogenetic tree for Bacillus methylotrophycus strain Ba1 and related species

3.3. Effect of incubation conditions on growth of Ba_1 strain

Temperature, pH and inoculum rate directly impact on growth of bacteria. The results showed that the Ba_1 strain was not much sensitive to temperature because it was adapted to grow and divide at a wide range of temperature from 20°C to 45°C. However, the optimum temperature was 30°C leading to the highest biomass yield was obtained, estimated to be OD_{600} = 2.35 (figure 3A).

In the pH ranges, at pH of 5.0, 6.0 and 7.0 were found to be suitable for growth of this strain and the optimum pH value was 6.0. When pH was adjusted to 3.0, 4.0 and 8.0, 9.0, the growth of the Ba_1 strain was inhibited (figure 3B). The results also showed that the optimum inoculum rate was 7% (v/v) (fig. 3C).

There are some differences between this study and Hoa et all studies that the optimum temperature and initial pH for *B. subtilis* growth were 35°C and pH = 7.0.

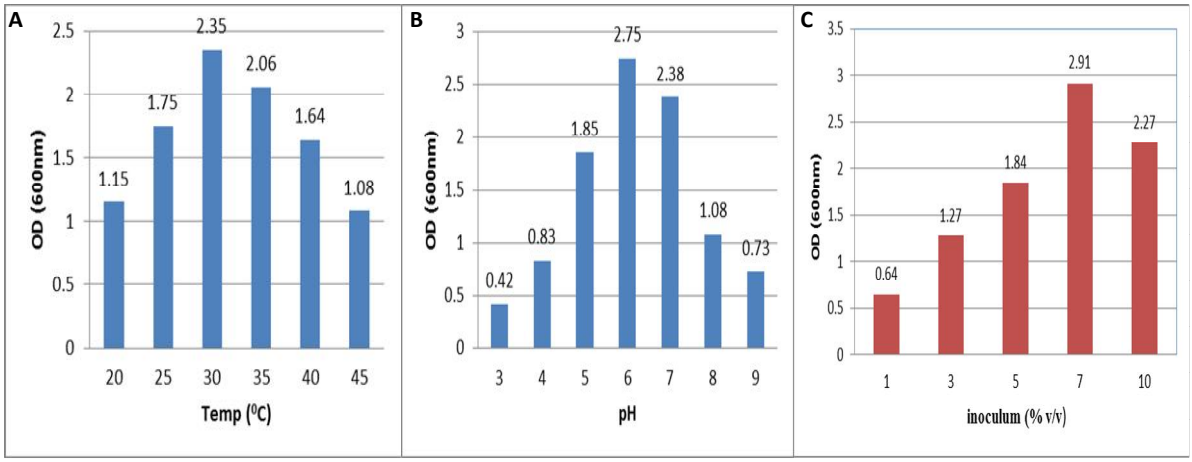

Figure 3. Effect of incubation conditions: temperature (A), pH (B) and inoculum rate (C) on growth of Ba1 strain

IV. CONCLUSION

From wastewater and sludge at craft village - Minh Hong-Bavi, Hanoi, Ba_1 strain was selected and identified as *Bacillus methylotrophycus*. This strain was expressed to be a useful candidate for wastewater treatment. It could use wide range of substrates, high efficiency moving of COD (90% COD was removed after 18 hours) and its activated sludge settled well with SVI = 120.4. The optimum culture conditions were identified at 30°C, initial medium pH = 6.0 and inoculum of 7% v/v and the maximum biomass yield was obtained, estimated to be OD_{600} = 2.91 after 24 hours incubation.

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ĐẶC ĐIỂM SINH HỌC VÀ KHẢ NĂNG ỨNG DỤNG CỦA CHỦNG VI KHUẨN BẢN ĐỊA *B. METHYLOTROPHYCUS* Ba_1 TRONG XỬ LÝ NƯỚC THẢI LÀNG NGHỀ CHẾ BIẾN TINH BỘT DONG RIỀNG

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TÓM TẮT

Nước thải từ làng nghề sản xuất và chế biến tinh bột dong riềng ô nhiễm hữu cơ ở nồng độ cao (tinh bột, cellululose, protein, xylan,..), đặc biệt là hàm lượng tinh bột sót sau khâu nghiền, lắng và lọc bột. Các thông số COD, BOD_5, SS, coliform,… vượt tiêu chuẩn cho phép hàng trăm lần. Để xử lý sinh học nước thải ô nhiễm hữu cơ theo định hướng kết hợp xử lý hiệu quả với tận thu giá trị ô nhiễm, nghiên cứu này quan tâm chọn lọc và ứng dụng các chủng vi sinh vật có năng lực chuyển hóa nhanh, đa dạng và hiệu quả các hợp chất hữu cơ thành sinh khối vi sinh vật. Sinh khối vi sinh vật cùng với bùn hoạt tính và các chất ô nhiễm không hòa tan còn lại trong nước thải sẽ được loại bỏ ngay trong quá trình xử lý nhờ vào khả năng kết lắng nhanh, hiệu quả của bùn hoạt tính. Từ nước thải và bùn thải sau chế biến dong riềng, tuyển chọn được chủng *Bacillus methylotrophicus* Ba_1 đáp ứng được giải pháp công nghệ xử lý hiếu khí có tách phân ly bùn hoạt tính ngay trong quá trình xử lý với các đặc tính: sử dụng đa dạng cơ chất (xylan; protein; tinh bột; cellulose), trong đó hoạt tính phân giải tinh bột của chủng Ba1 cao (D/d = 12,5). Chủng Ba_1 có khả năng làm giảm 90% COD sau 12 giờ, bùn hoạt tính lắng tốt với SVI = 120,4. Kết quả khảo sát điều kiện sinh trưởng tối ưu của chủng Ba_1 là ở 30^{o}C, pH 6 và tỷ lệ cấp giống 7%, sau 24 giờ sinh khối đạt OD_{600} = 2,91.

Từ khóa: *Bacillus methylotrophicus*, bùn hoạt tính, đặc điểm, tuyển chọn, xử lý nước thải.

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