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Ethanol production by mixed-cultures of *Paenibacillus* sp. and *Zymomonas mobilis* using the raw starchy material from sweet potato

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Abstract - The conversion of raw starchy materials from sweet potato into ethanol in a mixed-culture of an amylolytic bacterium, *Paenibacillus* sp. and different strains of *Zymomonas mobilis* were studied. Raw starchy material from sweet potato was hydrolyzed by *Paenibacillus* sp. to glucose, which is directly used by *Z. mobilis* for ethanol production. A mixed-culture of *Z. mobilis* ATCC 29191 and *Paenibacillus* sp. 9 yielded the highest ethanol concentration (6.89 g/l) in cultures of small volumes. The effects of medium pH (the best pH value were between 5.0 and 6.0) and the supplementation of the medium with glucose on ethanol production were also studied. In a large-scale fermentation, the final concentration of ethanol was 6.60 g/l, which corresponds to 23.24% of the theoretical yield of the ethanol from 50.0 g/l of the starch from raw sweet potato after a 120 h fermentation period.

Key words: bioethanol; mixed-culture; *Paenibacillus* sp.; sweet potato starch; *Zymomonas mobilis*.

INTRODUCTION

Starch is one of the most abundant polysaccharides in nature and represents an important biomass resource for bioethanol production. Brazil and US together account for about 60.0% of the world ethanol production exploiting sugarcane and corn, respectively (Chandel *et al*., 2007). China is by far the largest producer of sweet potato in the world, accounting for 85.0% of global production. In China, sweet potato is the fourth major staple crop and the second largest feed source in China. Its yield has remained fairly stable at 20-23 million tons per year since the 1970s (Huang, 2003). Sweet potato yields two to three times as much fermentable carbohydrate as field corn according to the US Department of Agriculture (Comis, 2008). This study also indicates that the root crops have a greater potential than corn grains as ethanol sources. Recently, fuel ethanol production based on the use of sweet potato has been growing in China (Comis, 2008; Schwartz, 2008). Sweet potato has been shown to be the least–cost effective option for ethanol production in China when compared to the cost of the bioethanol obtained from sweet potato, corn and cassava. Although China is the largest sweet potato producer in the world, sweet potato has not been efficiently utilized. As a candidate for bioethanol production, biomass utilization shows some advantages for the use of sweet potato in China.

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Zymomonas mobilis is a well-known ethanol-producing bacterium, and it has been historically used in tropical areas to make alcoholic beverages from plant sap (Skotnicki *et al.*, 1983). The advantages of *Z. mobilis* are its fast growth rate and high specific ethanol production rate. However *Z. mobilis* seems to show limitation since it metabolizes only a few types of sugars, such as glucose, fructose and sucrose (Swings and Deley, 1977). Traditionally, the production of industrial and fuel ethanol from starchy materials commonly involves a three-step process (Laluce and Mattoon, 1984). For the direct conversion of starchy materials to ethanol, the enlargement of the bacterial substrate range has been tried with the insertion of an of α-amylase gene from *Bacillus licheniformis* (Brestic-Goachet *et al*., 1990) and a glucoamylase gene from *Aspergillus niger* (Skotnicki *et al*., 1983) into *Z. mobilis* genome. Nevertheless the expression plasmids were unstable in *Z. mobilis* or showed low levels of activity (Skotnicki *et al*., 1983; Brestic-Goachet *et al*., 1990).

In order to lower the cost of the bioethanol production and to improve the ethanol yields from starchy materials, mixed-cultures of amylolytic organism and *Z. mobilis* have been used to convert starch or disaccharides into ethanol. The advantages of cultivating two or more microorganisms together (mixed-culture) over the cultivation of a single microorganism (pure culture) have been pointed out by many researchers (Tanaka *et al*., 1999). In the fermentation industry, mixed-culture systems have shown a great potential for applications in processes involving more than one reaction step (Kleerebezem and Loosdrecht, 2007). For example, cheap raw materials such as starch can be hydrolyzed and converted into ethanol in a mixed-culture system. This

type of process has been successfully used in the production of ethanol from starchy materials by co-cultivating two micro-organisms such as *Z. mobilis* and another microorganism that might be a yeast or bacterium. For example, a mixed-culture of *Endomycopsis fibuligera* NRRL 76 and *Z. mobilis* ZM4 was able to directly ferment cassava starch (22.5% w/v) into ethanol (10.5% v/v) more efficiently than a monocultures as described by Reddy and Basappa (1996). The conversion of starch into ethanol in a mixed-culture of *Saccharomycopsis fibuliger* and *Z. mobilis* was also studied (Dostálek and Häggström, 1983). Mixed-cultures of *Bacillus amyloliquefaciens* MIR-41 and *Z. mobilis* Flo-B3 showed a 2.5-fold increase in the α-amylase production, and a 20 times increase in the ethanol production when compared with pure cultures (Abate *et al*., 1999).

Paenibacillus is a soil bacterium able to produce a variety of hydrolytic enzymes including extracellular proteases, cellulases, pectinase, chitinases and even cyclodextrinases (Budi *et al*., 2000). As the bacterium *Paenibacillus* sp. 9, a mutant obtained in this laboratory, showed the ability to convert starch from sweet potato into glucose (unpublished results) in the present work, it was decided to establish the conditions for carrying out a mixed fermentation to obtain the direct saccharification and fermentation of the sweet potato starch into ethanol in a mixed-culture containing the new amylolytic strain of *Paenibacillus* and a strain of *Zymomonas mobilis*.

MATERIAL AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. *Zymomonas mobilis* was cultured without shaking in Rich medium (Goodman *et al*., 1982) at 30 °C for 36 h. *Paenibacillus* sp. was grown without agitation at 30 °C for 48 h on the following medium (g/l): glucose 10, $NaNO_3$ 2, NH_4Cl 1, KCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, K_2HPO_4 1.5, $FeSO_4{\cdot}7H_2O$ 0.025, pH7.0. The fermentation medium RMS50 was prepared to contain initially the following (g/l): sweet potato starch 50, yeast extract 10, KH_2PO_4 1, $(NH_4)_2SO_4$ 1, $MgSO_4{\cdot}7H_2O$ 1. All the media were sterilized in autoclave prior to use.

Fermentation assays. All fermentation assays were carried out in duplicate. Sets of eight 100-ml shaken flasks, containing 50 ml fermentation medium RMS50, were inoculated as follows: 2.5 ml *Paenibacillus* sp. 3 (flask A, as control); 2.5 ml *Z. mobilis* (flask B, as control); 2.5 ml *Paenibacillus* sp. 3, after 48 h of incubation 2.5 ml of different strains of *Z. mobilis* were added (flask C, D,

TABLE 1 - Strains used in this work

Strains	Source
Zymomonas mobilis CICC 10232	China Center of Industrial Culture collection
Zymomonas mobilis CICC 10225	China Center of Industrial Culture collection
Zymomonas mobilis IFFI 10225	China Center of Industrial Culture collection
Zymomonas mobilis ATCC 29191	China Center of Industrial Culture collection
Paenibacillus sp. 3	Wild type, this laboratory
Paenibacillus sp. 9	NTG (nitrosogranidine) – mutant strain from *Paenibacillus* sp. 3, this laboratory

TABLE 2 - Ethanol production by different strategies used to assay mixed-cultures of *Paenibacillus* spp. and *Zymomonas mobilis* in RMS50 medium at 30 °C for 120 h

Assay[a]	First inoculation	Second inoculation after 48 h (*Z. mobilis*)	Simultaneous inoculation	Glucose addition (4 g/L)	Initial pH	Ethanol (g/l)[b]
A	*Paenibacillus* sp. 3	-	-	-	7.0	ND[c]
B	*Z. mobilis* ATCC 29191	-	-	-	7.0	ND
C	*Paenibacillus* sp. 3	*Z. mobilis* IFFI 10225	-	-	7.0	3.89
D		*Z. mobilis* ATCC 29191	-	-	7.0	4.23
E		*Z. mobilis* CICC 10232	-	-	7.0	2.59
F		*Z. mobilis* CICC 10225	-	-	7.0	2.46
G	-	-	*Paenibacillus* sp. 3 and *Z. mobilis* IFFI 10225	-	7.0	3.35
H	-	-	*Paenibacillus* sp. 3 and *Z. mobilis* ATCC 29191	-	7.0	3.72
I	-	-	*Paenibacillus* sp. 3 and *Z. mobilis* CICC 10232	-	7.0	2.07
J	-	-	*Paenibacillus* sp. 3 and *Z. mobilis* CICC 10225	-	7.0	1.99
1	*Paenibacillus* sp. 9	*Z. mobilis* ATCC 29191	-	-	4.0	5.43
2			-	-	5.0	6.75
3			-	-	6.0	6.89
4			-	-	7.0	5.56
5	*Paenibacillus* sp. 9	*Z. mobilis* ATCC 29191	-	Added	4.0	4.96
6			-	Added	5.0	6.90
7			-	Added	6.0	5.69
8			-	Added	7.0	5.92
L	*Paenibacillus* sp. 9	*Z. mobilis* ATCC 29191	-	-	6.0	6.60

[a] L: represent a larger-fermentation as described in materials and methods. [b] Values are averages of two independent experiments. [d] ND, not detected (too low to be detected).

E and F); 2.5 ml *Paenibacillus* sp. 3 and 2.5 ml *Z. mobilis* were inoculated simultaneously (flask G, H, I and J).

The second fermentation assay was carried out according to a mixed-culture in flask C to J which yielded higher concentration of ethanol and *Paenibacillus* sp. 9 was used. The initial medium was adjusted to the following values pH: 4.0, 5.0, 6.0 and 7.0 (Table 2, assay 1 to 4). Another fermentation assay was also performed simultaneously by adding 4 g/l glucose in the initial medium at different pH values (Table 2, assay 5 to 8). Finally, a larger-scale fermentation was performed as follows: 1000 ml shaken flask contained 500 ml fermentation medium which was inoculated with *Paenibacillus* sp. 9 and *Z. mobilis* (Table 2, assay L).

Analytical methods. Ethanol produced was assayed using a GC103 chromatograph (Shanghai, China) equipped with a glass column (0.26 x 200 cm) filled with Porapak Type QS (80–100 mesh, Waters, Milford, MA) at 150 °C and a FID detector at 80 °C. N_2 was the carrier gas (30 ml/min). Reducing sugars were determined every day by the DNS method (Miller, 1959). Total reducing sugars were estimated by the same method after concentrated HCl hydrolysis (30 min at 100 °C, and a pH adjusted to 7.0 with NaOH).

Filter paper chromatography for samples from supernatant of the mixed-cultures. Twenty µl of fermentation broth were spotted on the Xinhua filter paper (Hangzhou Whatman-Xinhua Filter Paper, China) and analyzed by paper chromatography in a solvent system made of butanol-ethanol-water (5:3:2, v/v) .The migrated sugar products were visualized by dipping the paper in 1.0% NaOH aqua-ethanol, after it was sprayed with 16.0% $AgNO_3$-acetone (1:9, v/v). Then the filter paper was washed by immersing it for 30 min in a 6 M ammonia solution.

Scanning electron microscopy. Biomass collected by centrifugation at 10000 *x g* for 1 min in an Eppendorff microcentrifuge was washed twice with distilled water, dehydrated in 30.0, 50.0, 70.0, 80.0, 85.0, 90.0, 95.0, 100.0% ethanol gradient, and then air-dried, coated with gold and observed under an electron microscope.

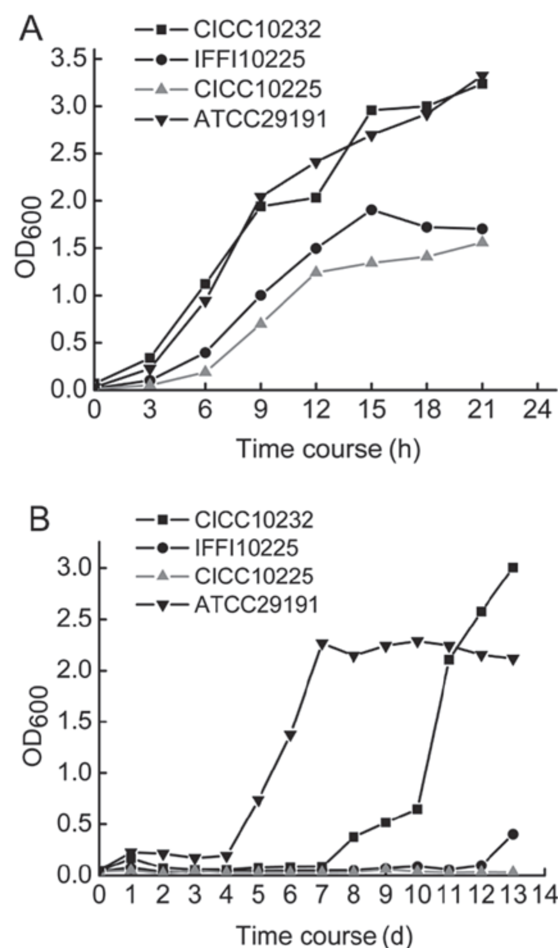

FIG. 1 - Growth curve of different *Zymomonas mobilis* strains under 10.0% of ethanol condition (initially added into the media) at pH 7.0 (A) and at pH 4.0 (B).

RESULTS AND DISCUSSION

Effect of different mixed-culture on ethanol production

Zymomonas mobilis has attracted considerable interest over the past decades as a result of its unique metabolism and ability to rapidly and efficiently produce ethanol from simple sugars (Rogers *et al*., 2007). It has shown a number of advantages over the existing ethanol-producing microorganisms and has emerged as a potential alternative to the yeast presently used in ethanol production (Panesar *et al*., 2006). Furthermore, a bacterium identified previously as *Paenibacillus* sp. in our laboratory was able to hydrolyze raw starch from sweet potato into glucose. So, the two strains (*Paenibacillus* sp. and *Z. mobilis*) were selected for setting up of a mixed-culture to directly ferment starch from sweet potato to ethanol. As shown in Table 2, a mixed-culture of *Z. mobilis* ATCC 29191 and *Paenibacillus* sp. 3 yielded higher ethanol concentration (4.23 g/l in 120 h, Table 2, assay D) than others after a 120 h fermentation period. Furthermore, when *Paenibacillus* sp. 3 was allowed to grow for 48 h before *Z. mobilis* was added (Table 2, assay C to F), the mixed-culture showed higher ethanol yields (2.46 to 4.23 g/l ethanol) than those obtained in cultures where both bacteria strains were inoculated at the same time given only 1.99 to 3.72 g/l ethanol (see Table 2, assay G to J). However, the best results were obtained when *Paenibacillus* sp. 9 was associated with *Z. mobilis* ATCC 29191 given ethanol yields which changed from 5.43 to 6.89 g/l (Table 2, assay 1 to 4). Maximal levels of ethanol were obtained between pH 5.0 and 6.0 (Table 2, assay 2 and 3). A higher ethanol level (6.6 g/l) was maintained at pH 6.0 when the reaction volume was increased (Table 2, assay L). The increased ethanol production in mixed-culture might be due to the rapid utilization of the glucose by *Z. mobilis* ATCC 29191 which was improved due to the secretion of amylolytic activity by the bacterial strain *Paenibacillus* sp. 9. As a good candidate for ethanol production, *Z. mobilis* ATCC 29191 displayed higher ethanol production and acid tolerant than other *Z. mobilis* strains (Fig. 1). Only the strain ATCC 29191 of *Z. mobilis* showed a fast growth at pH 4.0 as shown in Fig. 1B. At pH 7.0 (Fig. 1B) the strains of *Z. mobilis* CICC10232 and IFFI10225 showed the fastest growth and highest biomass yields. *Paenibacillus* sp. 9 is a NTG-mutant obtained from *Paenibacillus* sp. 3 and showed higher ability to digest starch (data not shown). Thus, *Paenibacillus* sp. 9 was used in the rest of the fermentation assays described below.

Effect of medium pH and initial glucose supplement on ethanol production

Generally, the optimum pH values for glucoamylase are 4.0-4.5 and for α-amylase 5.0-7.0 (Fang and Ford, 1998). The effects of medium pH and initial supplementation of the medium RMS with 4 g/l glucose are shown in Table 2 (assay 5 to 8). Ethanol produc-

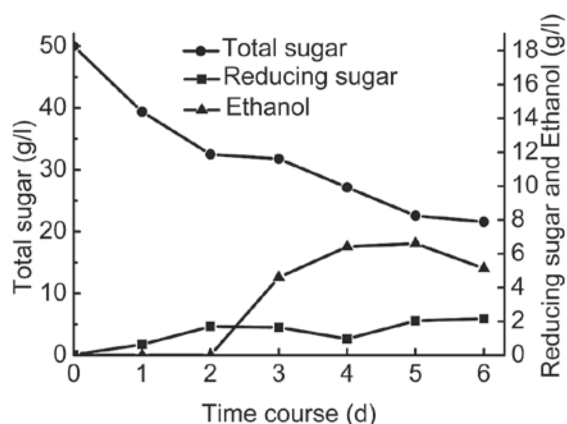

FIG. 2 - Large-scale fermentation of 5.0% raw sweet potato starch for 144 h at pH 6.0 in a mixed-culture of *Zymomonas mobilis* ATCC 29191 and *Paenibacillus* sp. 9. Values are averages of two independent experiments.

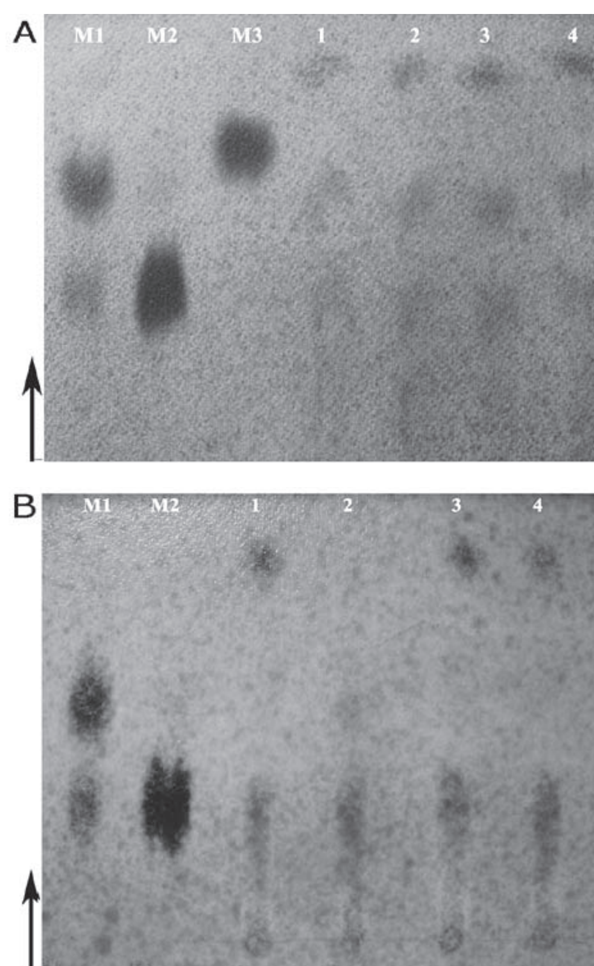

FIG. 3 - Fermentation products identified by filter paper chromatography in the supernatants of mixed cultures of *Paenibacillus* sp. 3 and *Zymomonas mobilis*. A: *Paenibacillus* sp. 3 after 48 h incubation in a starch-based medium (as control). B: Products of 120 h mixed culture of *Z. mobilis* and *Paenibacillus* sp. 3. Lane 1: *Z. mobilis* IFFI 10225; lane 2: *Z. mobilis* ATCC 29191; lane 3: *Z. mobilis* CICC 10232; lane 4: *Z. mobilis* CICC 10225. M1: a mixture of glucose and maltose used as standard; M2: maltose standard; M3: xylose standard. Black arrow indicates direction of the migration of sugar on the filter paper.

tion in the medium (pH 5.0 and pH 7.0) showed increase with the addition of initial glucose to the medium (Table 2, assay 2 and 6, assay 4 and 8). Glucose (4 g/l) was initially added to the medium RMS50 (pH 5.0), which yielded 2.07% increase in ethanol concentration (6.89 g/l in 120 h, Table 2, assay 6) than RMS50 (Table 2, assay 2). At pH 7.0, which yielded 6.47% increase in ethanol concentration when 4 g/l glucose added. However similar trend in terms of ethanol concentration wasn't observed in medium at pH 4.0 or pH6.0 with glucose supply (Table 2, assay 1 and 5, assay 3 and 7). These results indicated that the addition of 4 g/l glucose seem did not show any dramatic effect on ethanol production (Table 2, assay 5 to 8). In RMS50 medium supplied with glucose, the final reducing sugar concentration was higher than that in the unsupplemented medium (data not shown). An interested result was also observed when *Paenibacillus* sp. 3 was replaced with *Paenibacillus* sp. 9, and this led to an increase of 31.5% in the ethanol yield (Table 2, assay D and assay 4). The increased ethanol production in mixed-culture might be due to the rapid released glucose from raw starch by *Paenibacillus* sp. 9 (unpublished data).

Large-scale fermentation

Since no significant differences were observed when 4 g/l glucose was added initially to the RMS50 at pH 5.0 or pH6.0 keeping all the other variables constant (Table 2). In 72 h, the amount of ethanol produced was 4.60 g/1 when the medium contained 50.0 g/l of raw sweet potato starch (Fig. 2). However, when the fermentation time was increased to 120 h, the ethanol production was also increased to 6.60 g/l (Table 2, assay L, and Fig. 2), which is 23.24% of the theoretical yield of ethanol. Actually, when the fermentation time was increased to 96 h the ethanol production reached to 6.42 g/l. The amount of reducing sugar was 2.034 g/l (Fig. 2). The kinetics of ethanol production from starch by this strain in mixed-culture is shown in Fig. 2. Low amount of reducing sugar might be due to a low starch digestion efficiency or the rapid utilization of the glucose by *Z. mobilis*.

Filter paper chromatography of supernatant from mixed-cultures and a bacterial scanning electron microscopy

The reducing sugars in ferment culture were identified by filter paper chromatography. As shown in Fig. 3A, there are three types of reducing sugars (maltose, glucose, and an unknown reducing sugar) in the supernatant of the culture of *Paenibacillus* sp. 3 after 48 h incubation in a starch-based medium. However, in mixed-culture of *Z. mobilis* and *Paenibacillus* sp. 3 only two types of reducing sugars (maltose and the unknown reducing sugar) as shown in Fig. 3B. Major glucose may be consumed by the two strains, and used to produce ethanol by *Z. mobilis*.

Furthermore, for deep understanding the mixed-culture between *Z. mobilis* and *Paenibacillus*, we also performed assay of scanning electron microscopy. As shown in Fig. 4, raw starch was tightly bound by bacterial cells in mixed-culture of *Z. mobilis* ATCC 29191 and *Paenibacillus* sp. 9. Since both bacteria shows are rod shape, it is impossible to determine the ratio between the two strains with a reasonable degree of accuracy. There is starch particle remained after 72 h of incubation in the starch-based medium (Fig. 4).

Further research will be focused on the development of efficient processes for one step (such as SSF fermentation) bioconversion of starch into ethanol (Altintas *et al*., 2002). Methods reported for the fermentative production of ethanol from starch include (i) simultaneous saccharification and fermentation with a mixed culture of amylolytic and an ethanol-producing micro-

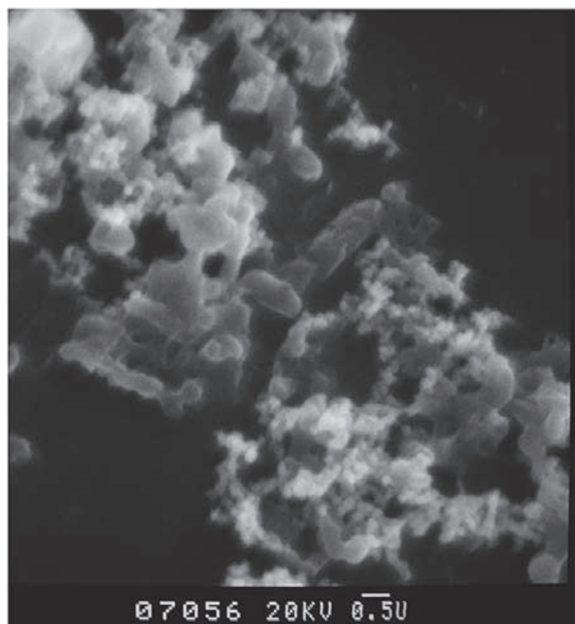

FIG. 4 - Scanning electron micrograph of a mixed inoculated with *Zymomonas mobilis* ATCC 29191 and *Paenibacillus* sp. 9 using a sample withdraw from the starch medium (RMS50) after 72 h of incubation in a starch-based medium. Initial concentration of starch used was 50 g/l.

organism (Dostálek and Häggström, 1983; Tanaka, 1986; Abouzied and Reddy, 1987; Han and Steinberg, 1987; Verma *et al*., 2000), (ii) use of both amylolytic enzymes from bacteria and a fermenting yeast together for the saccharification and fermentation of starch (Laluce and Mattoon, 1984; Hoshino *et al*., 1989, 1990), and (iii) addition of glucoamylase to the fermentation broth prior fermentation, which is a common practice in industry (Yamade and Fukushima, 1989). There is little information about fermentation using raw sweet potato starch with mixed cultures of microorganisms. This present type of fermentation process might transform raw sweet potato starch into a promising raw material for bioethanol production. In addition, the fermentation process should be optimized to enhance the ethanol production efficiency. Furthermore, this new isolated strain may be used in bioethanol production by introducing the ethanol pathway of *Z. mobilis* into it, or cloned its amylolytic enzyme gene into *Z. mobilis* to obtain an engineered organism able to directly converse raw starchy materials into ethanol.

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