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CONVERSION OF AGRICULTURAL WASTES TO ETHANOL AND ACETIC ACID BY CELLULOLYTIC ANAEROBIC BACTERIA

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ABSTRACT

An anaerobic spore-forming bacterium isolated from compost was the most potent cellulolytic strain among twenty strains screened from various sources in Thailand. It was identified as Clostridium josui *FERM P-9684. The optimum temperature and pH for growth were 45°C and 6.8, respectively. The strain could hydrolyse crystalline cellulose (Avicel), water hyacinth, BMC and rice straw which were mechanically ground without any chemical pre-treatment. Its high hydrolytic capability on tough cellulose contrasted with the almost negligible hydrolysis by* Ruminococcus albus *from the cow rumen. The main fermentation products were ethanol and acetic acid. The yields of ethanol and acetic acid obtained, especially from tough cellulose, were higher than those with* Coprococcus *species. These results show that* Clostridium josui *FERM P-9684 may be a promising anaerobe for the conversion of cellulosic materials to useful substances.*

INTRODUCTION

Cellulose is the cheapest and most abundant renewable resource for the long-term solution of raw material problems for energy, chemical and food production. The conversion of cellulosic materials to useful substances by microorganisms is one effective method of utilization of agricultural cellulosic wastes. The capacity to degrade cellulose is a character distributed among a wide variety of aerobic, facultatively anaerobic and strictly anaerobic bacteria and fungi. Although fungi are promising aerobes for degrading cellulose to glucose which can subsequently be converted to ethanol or other useful compounds by yeasts and bacteria,[1,2] the process requires at least two organisms or takes two steps. Thus, the fermentation abilities of anaerobic bacteria have recently received more attention,[3-5] since they directly convert cellulosic materials to ethanol or other chemicals within a few days. This simpler, so called single-step microbial process, is very attractive method of cellulose utilization.

Many strains of cellulolytic anaerobic bacteria have been reported[5-7] from various sources. Some strains are mesophilic while others are thermophilic. Usually thermophiles elaborate enzymes that are more active and more stable at high temperature than those produced by their mesophilic counterparts.[8] In addition, they have faster growth rates and higher rates of saccharification when compared with mesophiles.[9,10]

In this study, cellulolytic anaerobic bacteria from various sources in Thailand were isolated to determine their capability to convert natural cellulosic wastes to useful compounds..

MATERIALS AND METHODS

Microorganisms

For comparison, two reference strains of cellulolytic anaerobic bacteria, *Ruminococcus albus* from the cow rumen (supplied by Tohoku University, Sendai, Japan) and a *Coprococcus* species from Thai Compost, isolated by Sukhumavasi *et al.*[11] were used.

Samples for isolation of cellulolytic anaerobic bacteria were collected from various sources in Thailand, including compost, manure, silage, rumen fluid, rabbit caecum fluid, shipworms, termites and wood-attacking insects.

Cellulosic materials

Ball-milled cellulose (BMC) was prepared by ball-milling a 3% (w/v) aqueous suspension of pure cellulose (KC flock W-300 of Sanyo Kokusaku Pulp Co., Tokyo) for 3 days. Avicel, a microcrystalline cellulose, was the product of E. Merck Co., Darmstadt. Fresh water hyacinth (WH) was chopped and dried in the oven at 85°C for 72 h. Agricultural cellulosic wastes, such as rice straw and husks, corncobs, sawdust and bagasse, were not pretreated chemically. After drying, these cellulosic materials including WH were ground for 3 min by a vibrating sample mill (TI-100, Heiko Seisakusho, Ltd., Tokyo), sieved through a 100 mesh sieve (149μm) and used as the main carbon sources in the media. Cellobiose from Tokyo Kasei Co., Tokyo, was also used as a carbon source to give a clear liquid medium.

Other chemicals used were commercial products of the highest purity available.

Media preparation

Rumen fluid media were prepared by modifying the method described by Taya *et al.*[12] Briefly, the preparation procedure was as follows : a mixture of 7.5 ml each of mineral I (K_2HPO_4) and mineral II (KH_2PO_4, NaCl, $(NH_4)_2SO_4$, $MgSO_4 . 7H_2O$ and $CaCl_2$), 30 ml of rumen fluid, 20 mg cellobiose and 0.1 ml of 1% resazurin solution was stirred thoroughly. As the main carbon source, 0.5 g of one of the various cellulosic materials was added. The volume of the medium was adjusted to 100 ml by adding distilled water, and its pH was adjusted to 7.0 with 0.1 N HCl. After Na_2CO_3 (0.45 g) was added, the mixture was maintained at about 50°C. Then O_2-free CO_2 gas was bubbled through the medium for at least 30 min, to get rid of oxygen in the medium. This decreased the pH

of the medium to 6.8, so that the blue color of the medium turned to pink. Cysteine (2.5 mg) was then added. Finally, Na_2S 0.0025 g was added. Gas bubbling was continued until the pink color of the medium disappeared, showing a complete anaerobic condition. The medium was then dispensed into test tubes, 5 ml each, sealed with butyl rubber stoppers. All tubes were placed in a press rack to avoid dislodging of stoppers by the gas pressure building up within the tubes. The medium was then autoclaved at 110°C for 10 min. Solid media for use in the roll-tube method as described by Hungate[13] were prepared by adding agar (2%) to the liquid medium containing Avicel (1%).

Cultivation techniques

The cultivation of microorganisms was carried out in test tubes (12 mmϕ × 150 mm), Erlenmeyer flasks (250 ml) or in a jar-fermentor (2 l) under anaerobic conditions using O_2-free CO_2.

Isolation of cellulolytic anaerobic bacteria

For the isolation of cellulolytic bacteria, many samples were collected and each was inoculated into two tubes containing 5 ml of Avicel medium (one loopful per tube). One tube was incubated at 37°C and one at 45°C. After 45 h of incubation, cultures which produced discrete gas bubbles and solubilized cellulose, as judged by a decrease in the bulk of insoluble cellulose, were selected for isolation on solid media. Anaerobes with high cellulolytic activity were isolated by the roll-tube method. Colonies yielding large clear zones were then transferred individually into liquid medium by means of a sterile bent Pasteur pipette. This isolation procedure was repeated more than three times until the bacterial cells appeared homogeneous microscopically. According to this procedure, pure culture of potent cellulolytic strains were selected. The isolates were stored at -80°C as deep agar cultures in a medium containing 0.1% (w/v) Avicel as the main carbon source. They were transferred occasionally at 2 to 3-month intervals into fresh medium.

Cultivation of the selected anaerobes

A stock culture of each selected microorganism kept at -80°C was activated by inoculation into liquid Avicel medium incubated for 24 h at 45°C. Then, 0.2 ml of this activated culture was inoculated, in duplicate, into 5 ml of the basal medium containing the various cellulosic materials described above. Inoculated culture tubes were placed in a press test tube rack and incubated at 45°C for 7 days, after which the amounts of residual cellulose and products such as ethanol and volatile fatty acids in the supernatant broth were determined.

In order to follow the time course of cellulose degradation with an individual microbe, fifteen test tubes of 3-day old seed culture were transferred into 1,500 ml of medium (pH 7.0) in a 2-1 fermentor (Mini Jar Fermentor M-100, Tokyo Rikakikai Co., Ltd.). The percent of cellulosic substrate in the medium was 0.45%. During fermentation, the pH was not controlled but the temperature was held at 45°C. The agitation speed was set at 150 rpm.

When necessary, Silicone KM 72 (Shin-etsu Chemical Co., Tokyo) antifoam was added, one drop at a time.

RESULTS AND DISCUSSION

Cellulolytic and fermentation properties of the selected anaerobic bacteria in test tube cultures

Twenty pure strains of cellulolytic bacteria were isolated from the collected samples. Many from wood-attacking insects and from shipworms grew well at 37°C, while some others from compost grew well at 45°C. These differences in optimum growth temperature reflected the nature of the habitat since insects usually prefer temperatures lower than 40°C while the temperature in actively decomposing compost is usually higher than 40°C.[14] Amongst the twenty isolates, the extent of BMC solubilization was high in 6 strains which are shown in Table 1. Of these, strain III from compost was the most potent as judged by the decrease in the bulk of cellulose (9 $g.l^{-1}$). It could digest 6 g BMC per litre within 72 h. Regarding the reducing sugar, it is observed that nearly the same amount of sugar is released by all strains, which amount does not coincide with the cellulose degradation. This phenomenon may be the same as in the *R. albus* case in which most of the degraded glucose is not secreted from the cells into the culture broth.[15] After the water-insoluble cellulose is degraded to soluble cellulose or cellobiose, it is absorbed into the cells. Then, the cell-bound β-glucosidase degrades cellobiose to glucose which is assimilated in the cells and fermented to ethanol, volatile fatty acid, H_2 and CO_2. Thereafter, these products are secreted from the cells but glucose is not. Thus, the level of glucose in the broth is not related to the cellulose degradation as are the other products. Strain III was identified as *Clostridium josui* FERM P-9684. The details of its classification have been reported elsewhere.[16] *Clostridium josui* and Strain V were selected for further studies because of their outstanding ability to degrade cellulose.

The digestion of the microcrystalline cellulose, Avicel, by *C. josui* and Strain V was compared with that of the reference strains *Coprococcus* sp. and *R. albus* (Table 2). In 72-h cultivation, 4.0 $g.l^{-1}$, 3.4 $g.l^{-1}$ and 3.5 $g.l^{-1}$ of Avicel were degraded by *C. josui*, Strain V and *Coprococcus* sp., respectively. The main products from *C. josui* and Strain V were ethanol and acetic acid while the only product from *Coprococcus* sp. was ethanol. *R. albus* did not degrade Avicel.

Table 3 shows products in cultures of *C. josui* when BMC, Avicel, WH or rice straw was used as the main carbon source (4.5 $g.l^{-1}$). Within 7-days of cultivation, BMC was entirely degraded while other carbon sources remained not completely degraded. From all the carbon sources used, the main products were ethanol and acetic acid. There was negligible production of butyric and propionic acid. At 35 to 52 $unit.l^{-1}$, the CMC-degrading enzyme (CMCase) activities within these cultures were comparable.

Effect of temperature and pH on the growth of *C. josui* in a jar fermentor

Fig. 1 shows the relationship between specific growth rate and temperature when *C. josui* was cultivated in cellobiose liquid medium. The highest rate, 0.22 h^{-1} was obtained

at 45°C. No growth was observed below 25°C and above 65°C. These data indicate that the organism is a moderately thermophilic anaerobe. Fig. 2 shows the effect of initial medium pH on the specific growth rate of the strain. The rate was optimum at pH 6.8. At pH 4.5 and 8, the respective rates were 1/3 and 1/5 of the rate at the optimal pH.

Conversion of cellulosic materials to ethanol and acetic acid in a jar fermentor

Consumption of BMC and generation of fermented products by *Clostridium josui* was followed in a jar fermentor (Fig. 3 A). BMC (4.5 $g.l^{-1}$) was solubilized almost completely after 33 h of cultivation. The BMC degrading activity was comparable to that of *R. albus*[17] and that of a *Coprococcus* species.[11] The bacterial cell mass increased gradually to about 1.0 $g.l^{-1}$. The CMC hydrolytic activity of the culture supernatant was maximal at 40 h of fermentation. The accumulated amount of ethanol and acetic acid was around 1.6 $g.l^{-1}$ and the total volume of gas evolved per liter of culture broth was 800 ml.

When the crystalline cellulose, Avicel, was employed as the main carbon source (Fig. 3 B), degradation was about 90% after 43 h of cultivation, longer than that required for BMC degradation due to its toughness. CMC-hydrolysing activity in the culture supernatant reached a maximum after 48 h. Cell mass increased to 0.8 $g.l^{-1}$. The total gas evolved was 900 ml per liter of culture broth.

As for the degradation of rice straw (4.5 $g.l^{-1}$), it was almost completely degraded after 53 h of cultivation (Fig. 4A). A cell mass of about 0.8 $g.l^{-1}$ was harvested. CMC-hydrolysing activity reached a maximum after 58 h of cultivation. The total volume of gas evolved was 800 ml per liter of culture broth.

In the case of water hyacinth, cellulose was almost completely degraded within 33 h. The degradation rate was comparable to that of BMC, suggesting that water hyacinth was as soft as BMC. The cell mass increased to 0.9 $g.l^{-1}$ and the volume of gas evolved was 850 ml per liter of culture broth.

During the cultivation of *Clostridium josui* in a jar fermentor as described above, the pH of the broth decreased gradually from 7.0 to 6.4 in all cases. Ethanol and acetic acid were the major products released, while propionic acid, butyric acid and sugar were found only in trace amounts. These results coincided with those obtained from the test tube cultivations (Table 2). However, the product amounts in the jar fermentor were higher than those in the test tube cultures. This faster conversion was probably due to increased contact between the bacterial enzymes and the solid substrate caused by agitation. From BMC, ethanol and acetic acid were produced in nearly the same quantity (1.6 $g.l^{-1}$), while from Avicel, 1.2 and 0.8 $g.l^{-1}$ were produced, respectively. The yield of acetic acid from rice straw (1.0 $g.l^{-1}$) was higher than that of ethanol (0.6 $g.l^{-1}$). These differences probably arose from the differences in the degree of cellulose crystallinity and/or the components of the natural materials.

Since *Clostridium josui* can utilize chemically untreated Avicel and natural cellulosic materials to produce significant amounts of ethanol, acetic acid, single cell protein and cellulolytic enzyme, it is a promising anaerobe for conversion of both soft and tough agricultural cellulosic materials to useful products.

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บทคัดย่อ

จากการคัดเลือกแบคทีเรียที่สามารถย่อยสลายเซลลูโลสได้ดีในสภาพไร้ออกซิเจน จำนวน 20 สายพันธุ์ ซึ่งแยกจากแหล่งต่าง ๆ ในประเทศไทย พบว่า สายพันธุ์ที่มีศักยภาพสูงสุด เป็นแบคทีเรียมีสปอร์จากกองปุ๋ยหมัก ได้จัดจำแนกชนิดและให้ชื่อ เนื่องจากเป็น species ใหม่ว่า *Clostridium josui* FERM P-9684 แบคทีเรียสายพันธุ์นี้เจริญเติบโตได้ดีที่สุดที่อุณหภูมิ 45°ซ และความเป็นกรดด่าง (pH) ที่ 6.8 สามารถย่อยสลายเซลลูโลสรูปผลึก (Avicel), ผักตบชวา, เซลลูโลสรูปอสัณฐาน (BMC) และฟางข้าว ซึ่งเพียงบดให้ละเอียดได้โดยไม่ต้องผ่านกระบวนการย่อยสลายขั้นต้นทางเคมี อันสมรรถภาพในการย่อยสลายเซลลูโลสชนิดแข็งได้นี้เหนือกว่า *Ruminococcus albus* จากท้องโคซึ่งย่อยเซลลูโลสผลึกแข็งไม่ได้ ผลิตผลสำคัญที่ได้ในการหมัก คือ เอทิลแอลกอฮอล์และกรดน้ำส้ม ผลผลิต (yield) นี้สูงกว่าจาก *Coprococcus* species ผลจากการทดลอง บ่งชี้ให้เห็นว่า *Clostridium josui* FERM P-9684 เป็นแบคทีเรียที่อาจสามารถใช้เปลี่ยนวัสดุเซลลูโลสให้เป็นสารที่มีประโยชน์ได้ดีต่อไปในอนาคต

TABLE 1 Digestibility of BMC (9 $g.l^{-1}$) by six isolates after 72-h cultivation

Culture strain No.	Sources of organism	Digestibility	
		BMC degradation ($g.l^{-1}$)	Reducing sugar (as glucose) released ($g.l^{-1}$)
I	ship-worm	2	0.14
II	compost	4	0.21
III	compost	6	0.30
IV	compost	5	0.28
V	compost	5	0.31
VI	wood-borer insect	1	0.20

TABLE 2 Comparative data on digestibility of Avicel (4.5 $g.l^{-1}$) with three cellulolytic strains and the productivities after 72-h cultivation

Cultures	Avicel degradation (g/l)	Products	
		Ethanol (g/l)	Acetic acid (g/l)
Strain III *(Clostidium josui)*	4.0	0.9	0.5
Strain V	3.4	0.7	0.3
Coproccus species	3.5	0.8	ND
Ruminococcus albus	0	ND	ND

ND : not detected

TABLE 3 Products from various cellulosic materials by *Clostridium josui* in 5 ml of medium, after 7-day cultivation

Cellulose (4.5 g.l)	Consumed amount of cellulose	Remaining cellulose	Ethanol	Acetic acid acid	Propionic acid	Butyric acid	CMCase
	$g.l^{-1}$	$g.l^{-1}$	$g.l^{-1}$	$g.l^{-1}$	$g.l^{-1}$	$g.l^{-1}$	$u.l^{-1}$ **
BMC	4.5	0	0.8	0.8	ND	ND	46
Avicel	4.1	0.4	0.9	0.5	ND	ND	52
WH	4.2	0.3	0.6	0.9	trace	0.2	35
rice straw	4.0	0.5	0.2	0.3	trace	trace	41

ND : not detected

* Rumen fluid media containing 4.5 $g.l^{-1}$ of cellulose

** Unit of CMCase activity was defined as the fluidity, $(centipoise)^{-1}$, increased by 1 ml of enzyme solution catalysing hydrolysis of 5 ml of 1% CMC solution in 0.05 M phosphate buffer pH 6.8 at 37°C in 1 min

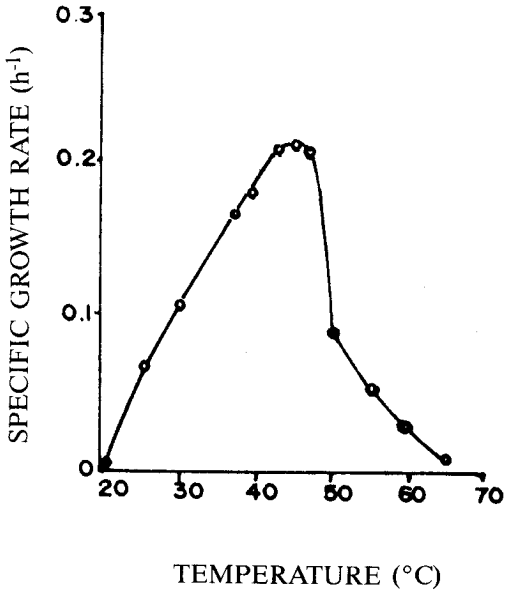

Fig. 1 Effect of temperature on the growth of *Clostridium josui* in cellobiose liquid medium (initial pH 6.8).

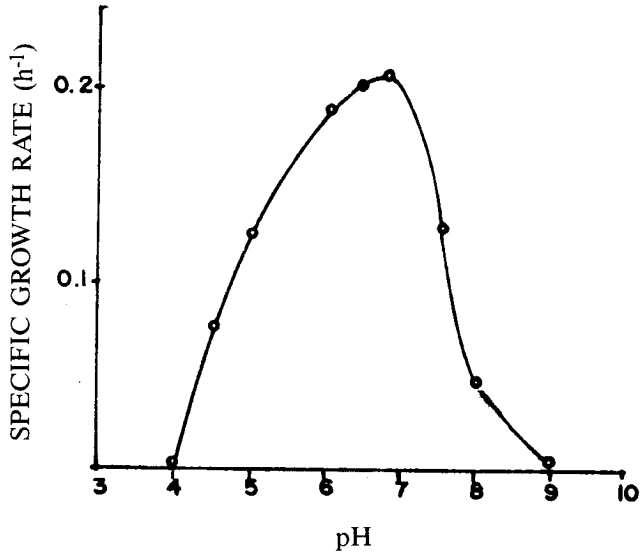

Fig. 2 Effect of initial pH on the growth of *Clostridium josui* in cellobiose liquid medium at 45°C.

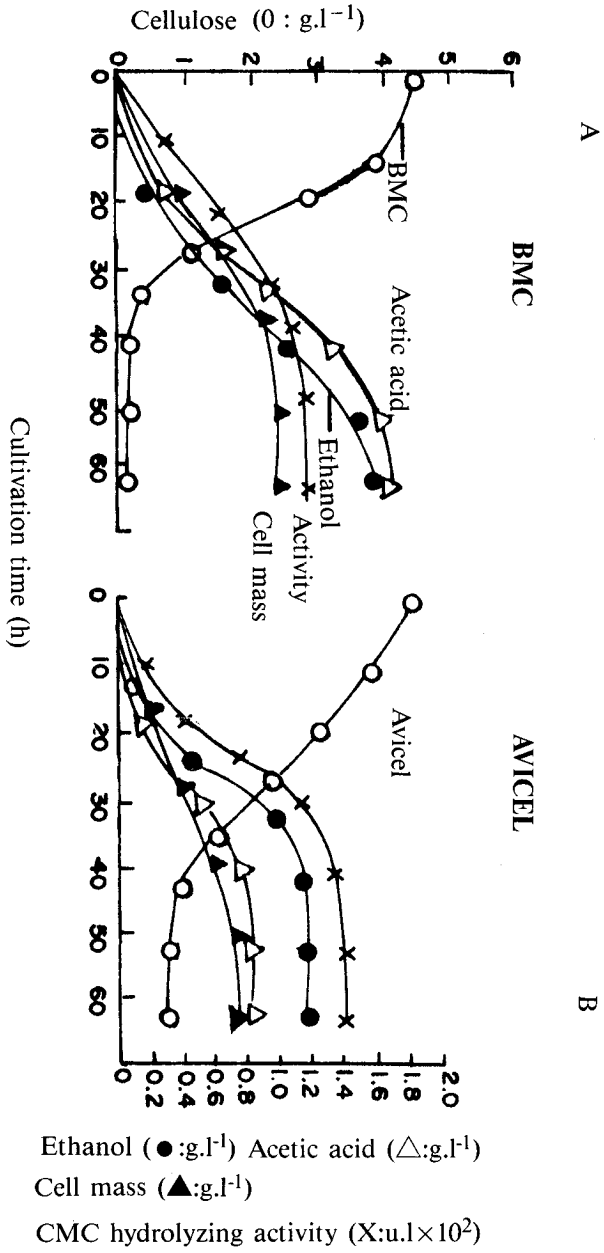

Fig. 3 Time coures of degradation of BMC and Avicel by *Clostridium josui*. The cultivations were carried out in a 2-l jar fermentor.

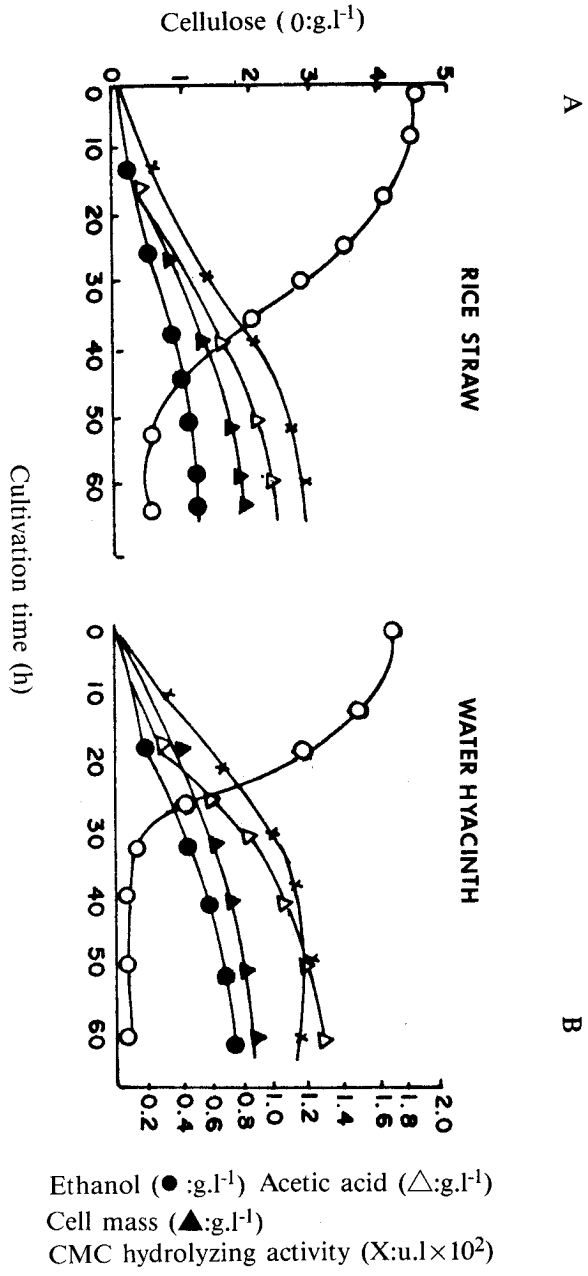

Fig. 4 Time courses of degradation of rice straw and water hyacinth by *Clostridium josui*. The cultivations were carried out in a 2-l jar fermentor.