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Fermentation of Xylose to Glycerol by *Rhizopus javanicus*

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ABSTRACT

Glycerol production from xylose fermentation using *Rhizopus javanicus* (ATCC 22581) has been investigated in shake flasks. The medium composition (xylose concentration, nitrogen sources), aeration rate, and temperature have been found to affect the accumulation and yield of glycerol. Some of these effects are explained in terms of the critical parameters, osmotic pressure, and dissolved oxygen levels in the medium. Relatively high glycerol yields and concentrations have been obtained at high sugar concentration with high level of aeration at room temperature. The addition of polyethylene glycol or sulfite can improve the yield and accumulation of glycerol.

Index Entries: *Rhizopus javanicus*; xylose; glycerol; xylose fermentation; glycerol production.

INTRODUCTION

Glycerol, an important polyhydroxy alcohol, has been traditionally produced as a byproduct of soap and fatty acids industry (*1*). After World War II, processes of glycerol chemical synthesis were developed (*2*). Since then, glycerol has found wide applications in many chemical and plastic industries. The largest amount goes into the manufacture of synthetic resins, ester gums, drugs, cosmetics, and toothpastes. The chemical route is not a viable process in the long run, since it is using up the limited depleting reserves of crude petroleum. The biochemical route may compete with the chemical route because the former is based on

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renewable raw materials (*1*). Hemicellulose is one of the most readily available renewable carbohydrate-rich resources that can be hydrolyzed easily to produce a mixture of monosaccharides, which contains about 70–80% xylose (*3,4*). The fermentation of xylose to glycerol by microorganisms is one of the promising and potentially viable processes for converting xylose from hemicellulose into marketable chemicals. Many processes involving the use of yeasts to ferment glucose to glycerol have been developed (*5–8*), but the fermentation with filamentous fungi that can ferment xylose to glycerol was not tried. It is here reported that the fungus *Rhizopus javanicus* is capable of fermenting xylose to glycerol. The conditions favoring the production of glycerol from xylose were investigated in shake-flask experiments using this fungus. The effect of nitrogen sources, sugar concentration, osmotic pressure, aeration, and temperature known to be important variables in regulating fungal fermentations were examined. Other factors evaluated included the effects of polyethylene glycol (PEG), sulfites, and sodium chloride.

MATERIALS AND METHODS

Microorganism

A *R. javanicus* strain (ATCC 22581) was obtained from the American Type Culture Collection.

Media and Culture Conditions

The strain was maintained on yeast extract–malt extract (YM; Difco) agar slants with 2% D-xylose as the carbon source. The cultivation medium consisted of the following, per liter: D-xylose, 50 g; urea, 2 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $ZnSO_4 \cdot 7H_2O$, 0.088 g; and yeast extract, 3 g; pH 6.8. The fermentation medium was similar, except that the xylose concentration was varied from 50 to 250 g/L. The fungal spores were collected from the agar slant culture by washing with sterile water to make a spore suspension before inoculation.

Inocula were prepared by cultivating the fungus in 250-mL Erlenmeyer flasks containing 100 mL of media. The fungus was grown under aerobic conditions at 30°C and 218 rpm. After 3 d of incubation, cells were harvested by filtration through milk filter paper. The mycelial mat was then resuspended in 250-mL Erlenmeyer flasks containing 100 mL media for batch fermentations. Whenever the medium composition was different, it has been indicated. Fermentations were conducted in a New Brunswick G27 controlled-environment incubator rotary shaker operating at 218 rpm and 30°C, unless otherwise indicated.

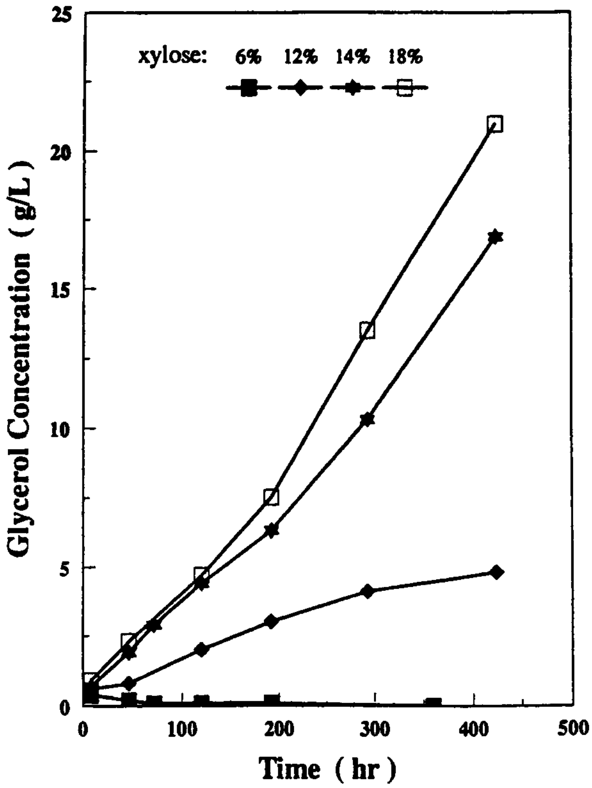

Fig. 1. Effect of xylose concentration on glycerol production by *R. javanicus*.

Analysis

Xylose, glycerol, and other products were determined by an isocratic HPLC system (Hitachi Instrument, AS-4000) equipped with an Aminex HPX-87H column (Bio-Rad, Melville, NY). The mobile phase was 0.01*N* H_2SO_4 at a column temperature of 60°C and a flow rate of 0.8 mL/min. ^{13}C NMR spectroscopy of the fermentation broth confirmed glycerol to be a major product under the conditions of experimentation.

RESULTS AND DISCUSSION

Effect of Sugar Concentration

Of the nutritional factors to be considered in the production of polyhydric alcohols by *R. javanicus* sugar concentration is probably the most important. The effect of sugar concentration was studied in the range of 5–18% w/v. Figure 1 shows the glycerol production at four different initial xylose concentrations. As the initial concentration of xylose was raised

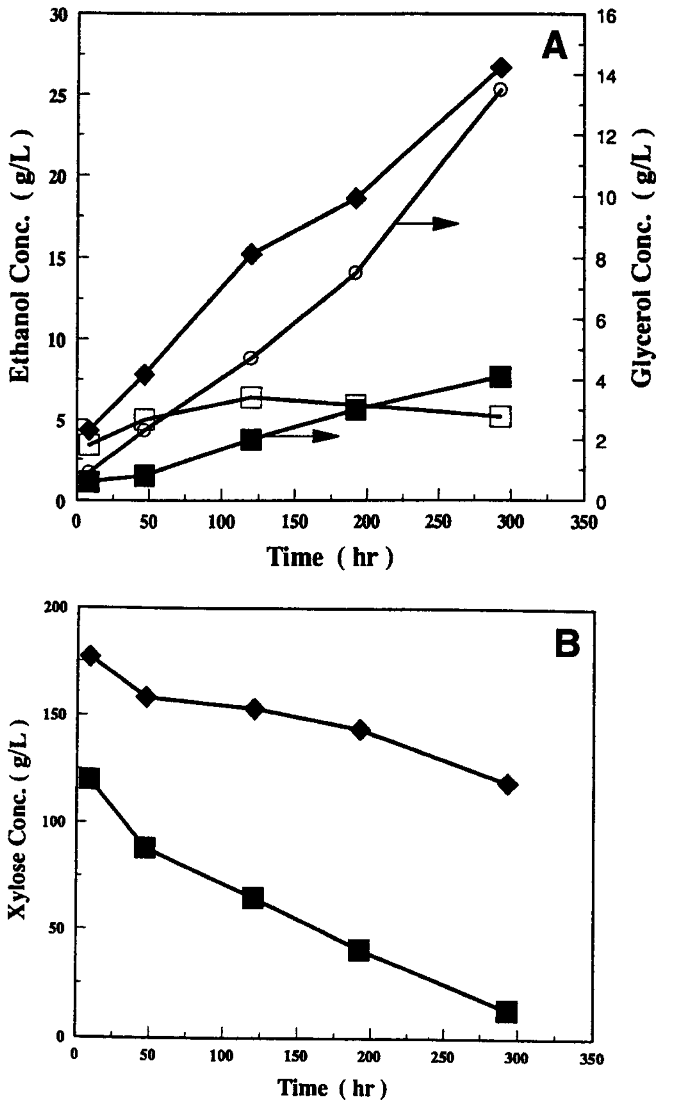

Fig. 2. (A) The productions of glycerol and ethanol at different initial xylose concentrations by *R. javanicus* (◆) Ethanol at 12% w/v xylose; (□) ethanol at 18% w/v xylose; (○) glycerol at 18% w/v xylose; (■) glycerol at 12% w/v xylose. (B) Xylose consumption at different initial concentrations during glycerol production by *R. javanicus*. (◆) Xylose, 18% w/v; (■) xylose, 12% w/v.

from 6 to 18%, the final concentration of glycerol was raised from 0 to 22 g/L. In contrast to this, ethanol produced in the medium declined with increasing sugar concentration (Fig. 2A). Xylose was consumed continuously during the course of fermentation and at low concentration was faster depleted than at high concentration (Fig. 2B). However, the glycerol yield (based on the sugar consumed) at low xylose concentration was poor. Raising initial xylose concentration from 12 to 18% increased yield

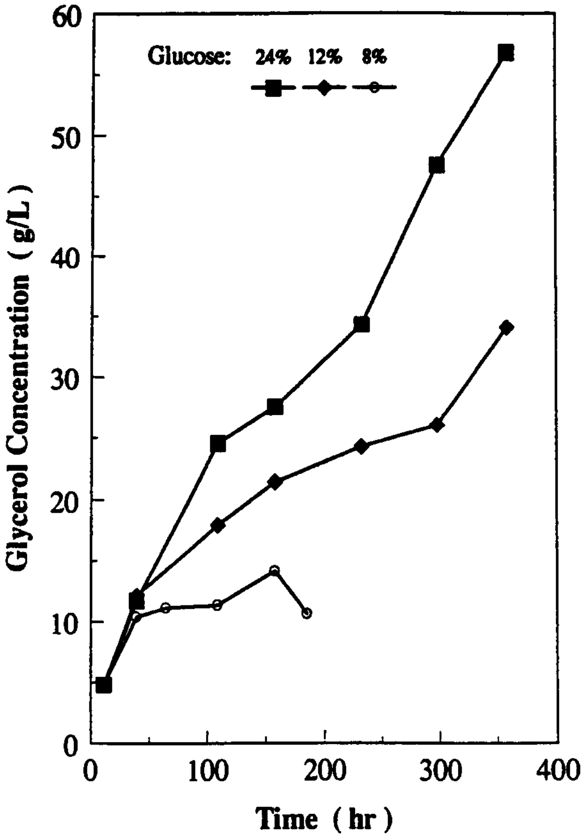

Fig. 3. Effect of glucose concentration on glycerol production by *R. javanicus*.

of glycerol from 4 to 29% on the basis of xylose consumed. Analysis by HPLC and ^{13}C NMR spectroscopy indicated that glycerol was the major product, and only small amounts of ethanol were produced if the medium contained initially a high xylose concentration. Similar observation was made by using glucose as a carbon source (Fig. 3). The requirement for relatively high initial sugar concentration is owing to the desirable effects of osmotic pressure on glycerol production. Accumulation of polyols is directly related to water activity relations of sugar-tolerant strains (*9*). Since higher glycerol yields were obtained with *R. javanicus* at higher sugar concentrations, further investigations were carried out using an initial xylose concentration around 17%. The result was that *R. javanicus* withstands higher sugar concentrations, which to some extent minimizes the chances of contamination.

Effect of Polyethylene Glycol

It has been reported that a decrease in water activity in the medium could be achieved by adding polyethylene glycol (PEG), and xylose assimilation and cell growth are not affected by the presence of PEG

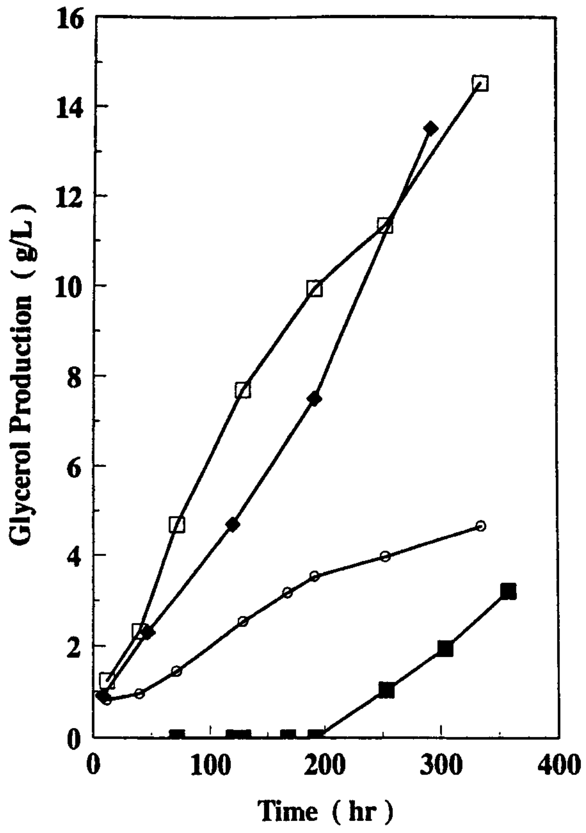

Fig. 4. Effect of PEG on glycerol production from xylose fermentation by *R. javanicus*. (□) PEG_{400}, 15% w/v; xylose, 11.5% w/v; (◆) no PEG; xylose, 17.7%; (○) PEG_{200}, 15% w/v; xylose, 11.5%; (■) no PEG; xylose, 10.4% w/v.

(*10,11*). In fermentations with the halotolerant alga *Dunaliella parva*, the addition of PEG has resulted in four times higher glycerol yields than with sodium chloride (*10*). In our preliminary experiments, it was noticed that with a high initial sugar concentration, a significant amount of unfermented sugar was left, and to elucidate this, the effect of PEG was investigated. PEG with average mol wt of 400 (PEG_{400}) and 200 (PEG_{200}) was chosen for this study. It was observed that better production rate and yield of glycerol were reached at low sugar concentration by addition of PEG (Fig. 4; Table 1). When 15% of PEG_{400} was in the medium, the final glycerol concentration was raised from 3.2 to 14.5 g/L, and the yield of glycerol was increased from 4.7 to 16.7% as compared to the one without PEG. The production rate of glycerol with the addition of PEG_{400} was higher than the one with elevated sugar concentration (18%) and no PEG addition during the first 250 h of fermentation. The improvement made by PEG_{400} was more significant than by PEG_{200}. Only slightly better yield was achieved by the addition of PEG_{200} (15% in the medium).

Table 1
Effect of Polyethylene Glycol on Glycerol Production from Xylose Fermentation

Initial xylose concentration, g/L	Average molecular weight of PEG[a]	PEG concentration, %	Fermentation time, hr	Glycerol concentration, g/L	Glycerol yield, %
104	NA[b]	NA	358	3.2	4.7
115	200	15	335	4.7	5.7
115	400	15	335	14.5	16.7
178	NA	NA	293	13.5	29.0

[a]PEG = polyethylene glycol.
[b]NA = not applicable.

An addition of PEG to substitute about half of initial sugar concentration was required for a significant glycerol production, which in turn lowered the amount of residual sugar from 120 to 35 g/L in the fermentation broth. The influence of PEG could be the result of the decrease of water activity (the increase of osmotic pressure), which is generally known to benefit glycerol production.

Effect of NaCl

An attempt to increase glycerol production was made by adding sodium chloride at the range 3–12% in the medium. It was noted that sodium chloride did not affect xylose fermentation, and *R. javanicus* could not utilize xylose. It is suggested that *R. javanicus* is sensitive to the sodium chloride presence.

Effect of Sulfite

The sulfite steering process for glycerol production was one of the first processes developed by Connstein and Lüdecke to meet emergency requirements of glycerol in Germany during World War I (*12,13*). The basis for this process was first suggested by Neuberg and coworkers (*14,15*). The reaction is believed to involve the fixation of acetaldehyde by sodium bisulfite ($NaHSO_3$) forming the complex $CH_3CHO{\cdot}NaHSO_3$:

$$\underset{\text{acetaldehyde}}{CH_3CHO} + NaHSO_3 \rightarrow \underset{\substack{\text{acetaldehyde-sulfite}\\ \text{complex}}}{CH_3CHO{\cdot}NaHSO_3} \quad (1)$$

In the absence of sodium bisulfite, acetaldehyde is reduced to ethyl alcohol during the glycolysis of sugars by *Saccharomyces cerevisiae*, but when acetaldehyde, a hydrogen acceptor, is fixed, the other triose produced from the hexose in the Embden-Meyerhof-Parnas (EMP) pathway acts as the main hydrogen acceptor and is reduced to glycerol. If all of the acetaldehyde produced is fixed by bisulfite, the stoichiometry of the reaction will be as follows:

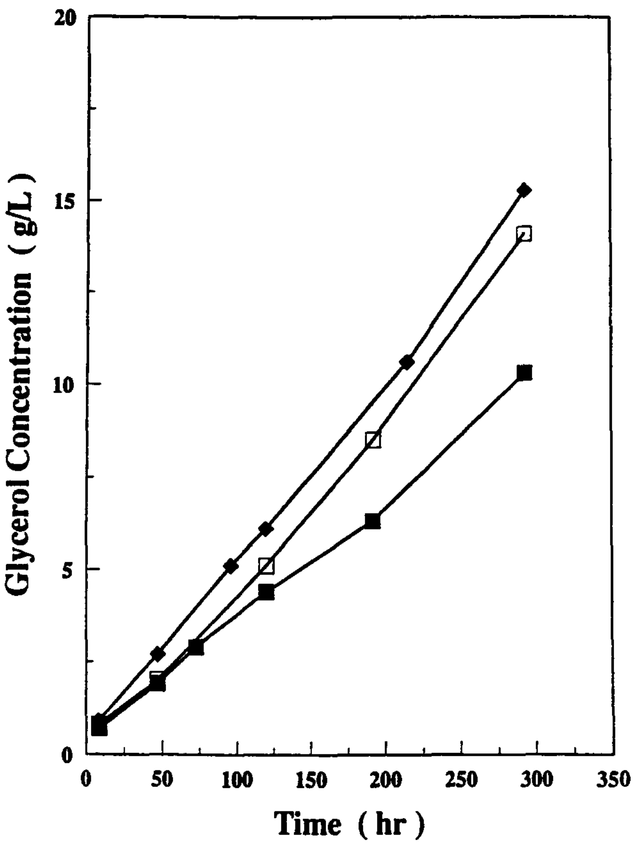

Fig. 5. Effect of sulfite on glycerol production from xylose fermentation by *R. javanicus*. (■) Control, no sulfite; (◆) 2.4% w/v Na_2SO_3; (□) 2.4% w/v $(NH_4)_2SO_3$.

$$\underset{\text{glucose}}{C_6H_{12}O_6} + NaHSO_3 \rightarrow \underset{\text{acetaldehyde-sulfite complex}}{CH_3CHO{\cdot}NaHSO_3} + \underset{\text{glycerol}}{C_3H_8O_3} + CO_2 \quad (2)$$

Therefore, it is possible that higher glycerol production and better yield could be achieved by the addition of sulfite. In a preliminary study, both sodium sulfite and ammonium sulfite were used as steering agents. The metabolism of the fungal cells was shifted from ethanol formation to glycerol formation by adding 2.4 g of sodium sulfite or ammonium sulfite into 100 mL of fermentation medium (containing 14% xylose). The sulfite was added in small portions to minimize inhibitory effects on the fungal metabolism. It was found that the sulfites had made a significant difference in glycerol production rate (Fig. 5). It was also observed that less ethanol was formed (data not shown), and the yields of glycerol were increased from 15.1 to 30.3% in the presence of sulfite. Although there was an insignificant difference between the effects of sodium sulfite and ammonium

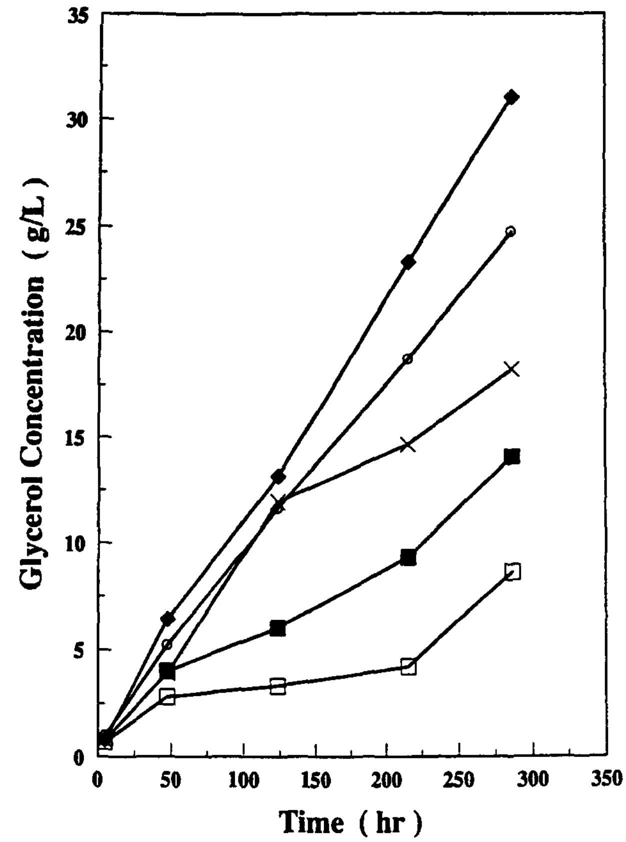

Fig. 6. Effect of nitrogen source on glycerol production from xylose fermentation by *R. javanicus*. (◆) Asparagine; (○) urea; (×) $NH_4H_2PO_4$; (◆) $NaNO_2$; (■) $NaNO_3$.

sulfite, slightly better glycerol production rate and yield increases were obtained by the addition of sodium sulfite than ammonium sulfite. Analysis by HPLC confirmed the formation of acetaldehyde-sulfite complex.

Effect of Nitrogen Source

The source and concentration of nitrogen greatly influence the yield of polyhydric alcohols (5). Therefore, preliminary experimentation was carried out to investigate the effect of the nitrogen source on glycerol formation by *R. javanicus*. It was found that media containing amino acids, such as asparagine and glycine, gave a better production rate than urea, sodium nitrite, sodium nitrate, ammonium nitrate, ammonium sulfate, ammonium chloride, and ammonium phosphate (Fig. 6). The production rate and yield of glycerol with sodium nitrite were poor. Relatively high yields of glycerol were achieved by using asparagine and ammonium chloride as nitrogen source (Table 2). During further studies, urea was

Table 2
Effect of Nitrogen Sources on Glycerol Production from Xylose Fermentation by *R. javanicus*

Nitrogen source	Nitrogen source's concentration, %	Final glycerol concentration, g/L	Glycerol yield, %
Glycine	0.500	25.9	33.1
Asparagine	0.500	27.3	37.5
Urea	0.200	22.0	28.4
$NaNO_2$	0.460	7.8	24.8
$NaNO_3$	0.566	12.2	33.0
NH_4NO_3	0.267	19.8	33.3
$(NH_4)_2SO_4$	0.440	20.8	30.6
NH_4Cl	0.356	23.6	36.0
$NH_4H_2PO_4$	0.766	17.1	32.9

selected as a nitrogen source. It was found that the urea concentrations had no significant effect on final glycerol concentrations. Yeast extract was also tried as a nitrogen source, and the production rate and yield of glycerol were higher when both urea and yeast extract were present than when urea or yeast extract was used alone (data not shown).

Effect of Aeration

Aeration is important in obtaining good glycerol production rate and yield. Figure 7 shows the effect of aeration on glycerol production. Low aeration resulted in very low glycerol production rate and yield, whereas increase in aeration from 155 to 265 rpm elevated the yield of glycerol from 23.4 to 33.1% with a concomitant decrease in ethanol formation and fermentation time reduction. The increase in the yield owing to the increase in the shaker speed is likely the result of increase in the aeration, which, however, has not been precisely determined. Results suggest that oxygen supply is essential for improvements in glycerol production by *R. javanicus*, and adjustment of oxygen levels available to the culture plays a significant role in polyhydric alcohols production. The actual oxygen requirement for maximum yield is variable and probably depends directly on the density of the population of cells in the culture. This is, in turn, influenced by other environmental and nutritional conditions (5).

Effect of Tempterature

Figure 8 depicts the effect of temperature on glycerol production from xylose fermentation by *R. javanicus*. Room temperature (25°C) gave a faster glycerol production rate than at 30 and 35°C; 35°C is a little better than 30°C in production rate. This can be partly attributed to the evapora-

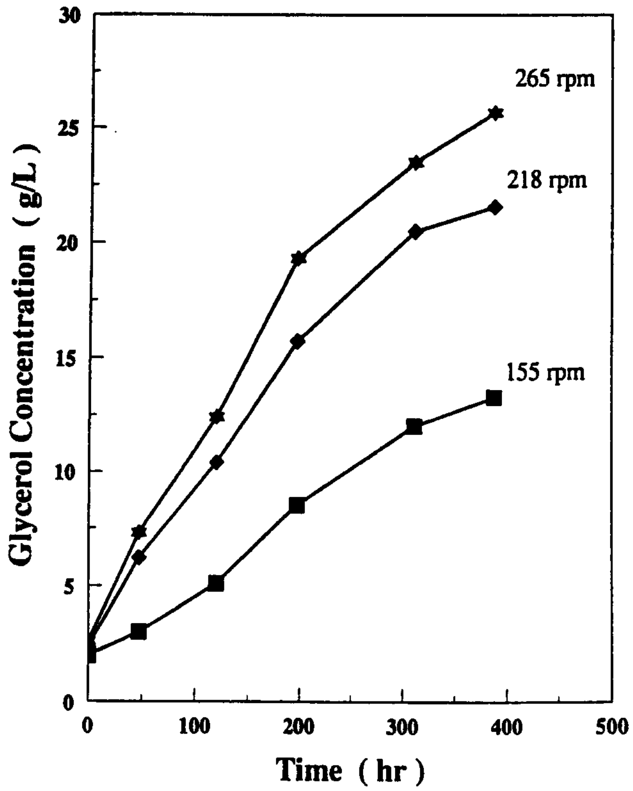

Fig. 7. Effect of aeration on glycerol production from xylose fermentation by *R. javanicus*.

tion at high temperature, which, in turn, increased sugar concentration in the fermentation broth, and benefited glycerol production. It was found that cell viability was greatly reduced at 35°C. The yields within the temperature range 25-35°C were between 23.3 and 25.8%.

CONCLUSIONS

In this study, it was demonstrated that glycerol production from xylose fermentation by *R. javanicus* is affected by sugar concentration. The fungus gave better accumulation and yield of glycerol in the presence of higher sugar concentration.

Improved yield of glycerol could be reached at lower sugar concentration by adding PEG of certain average molecular weight related to decreased/increased water activity (increased/decreased osmotic pressure). Glycerol yield can be increased by adding sulfite during fermentation, which forms a complex with acetaldehyde, thus increasing $NADH_2$ availability for the reduction of dihydroxyacetone phosphate to form glycerol (*16*).

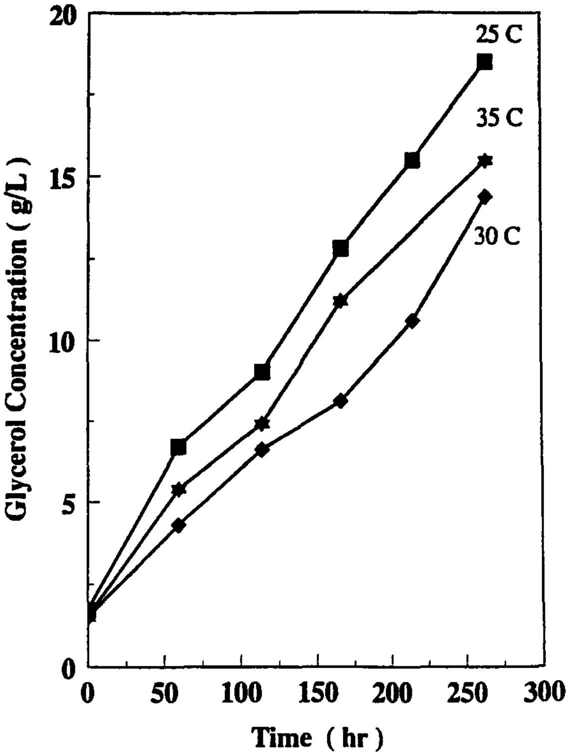

Fig. 8. Effect of temperature on glycerol production from xylose fermentation by *R. javanicus*.

The accumulation and yield of glycerol are also dependent on nitrogen source, aeration rate, and temperature. High level of aeration and room temperature (25°C) are beneficial for the production of glycerol. Further studies are needed to optimize the conditions for the production of glycerol from xylose fermentation by *R. javanicus*.

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