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Factors Affecting Glycerol Production by *Pichia farinosa* Under Alkaline Conditions

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ABSTRACT

Glycerol is an important and valuable chemical that can be produced from renewable resources by fermentation. The desirable features of a successful process are high values of substrate conversions and high yields and concentrations of glycerol in the product broth, coupled with rapid fermentation cycles. Of the various osmophilic and nonosmophilic yeasts tested for their ability to produce glycerol in the presence and absence of steering agents (sodium sulfite or sodium carbonate), an osmophilic yeast *Pichia farinosa* (ATCC 20210) was found to give attractive yields. Important variables influencing glycerol production by this strain under alkaline conditions using sodium carbonate have been investigated. A rapid fermentation (less than 120 h), coupled with high glycerol yields (45%), has been obtained.

Index Entries: Osmophilic yeasts; nonosmophilic yeasts; aeration; cell recycle; glucose utilization; alkaline pH; inoculum size; glycerol production, factors affecting.

INTRODUCTION

Pasteur (*1*) in 1858 first reported glycerol as a minor product formed during the manufacture of beer and wines. Later studies of Neuberg et

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al. (2) on the fermentation of sugars by yeasts in the presence of sulfites made possible the production of glycerol as a major product of fermentation. Subsequently, it led to an industrial scale of operation during the first world war by Connstein and Ludecke (3) and Cocking and Lilly (4). Eoff et al. (5) developed an alkaline process using sodium carbonate on a pilot-plant scale. All these studies were with nonosmophilic yeasts, and the maximum glycerol yields were in the range of 20–25%, based on the sugar. Onishi (6) reported that with osmophilic yeasts excellent yields of glycerol (40–45%, based on the sugar) could be obtained in the absence of steering agents [sodium sulfite or sodium carbonate, which steer the normal ethanol fermentation to glycerol fermentation via Embden-Meyerhof (EM)-pathway] in about 240 h. None of the above processes were used for commercial manufacture after the second world war, partly because of the availability of inexpensive petroleum based processes and partly because of low yields and productivities and recovery problems associated with the fermentation processes. However, with rapidly depleting petroleum reserves and consequent high prices, the fermentation route for glycerol production has now assumed great importance (7–9). Earlier we reported (10) that with *Pichia farinosa*, relatively high glycerol yields and concentrations, coupled with rapid fermentations, could be obtained in an alkaline medium. These results are superior to those of Onishi (6), with respect to fermentation times. A further systematic study was undertaken to give pertinent information toward evolving an economical process for glycerol production. This paper reports on studies of the following variables: effect of sodium carbonate or sodium sulfite; age and size of inoculum; degree of aeration; initial glucose concentration; urea and yeast extract; C/N ratio; sources of sugar and nitrogen; and effect of temperature. Also, the effects of sterilization and cell recycle on the product yields and fermentation rates have been investigated. All of these experiments were carried out in shake flasks under alkaline conditions, using sodium carbonate.

MATERIALS AND METHODS

Cultures

The yeast cultures were from the National Collection of Industrial Microorganisms (NCIM, Poona) and the American Type Culture Collection (ATCC, USA), and the cultures were maintained on malt extract (0.3%)–glucose (2%)–yeast extract (0.3%)–peptone (0.5%) (MGYP)-agar slants.

Shake Flask Experiments

Inoculum Preparation

A loopful of culture from the stock agar was transferred to a boiling tube containing 10 mL of MGYP media and grown for 24 h on a rotary

shaker at 180 rpm. This was then transferred to a 500-mL shake flask containing 90 mL of MGYP media, allowed to grow on the shaker for 24 h, centrifuged, and then used for inoculum.

Medium and Inoculum

The fermentation medium consisted of the following, in g/L: glucose, 300; yeast extract, 1.0; urea, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 1.0; and $CaCl_2 \cdot 2H_2O$, 0.1. Wet cells corresponding to 0.5 g dry cell weight (DCW) were suspended in 5 mL of the medium and inoculated into 500-mL shake flasks containing 80 mL of the above medium and incubated at 30°C on a rotary shaker at 180 rpm.

Analytical Procedures

The gluclose concentration was measured with glucose oxidase using a glucose analyzer (Yellow Springs Instruments, USA). Total polyol concentration was measured by the method of Neish (*11*) and reported as glycerol. Ethanol concentration was measured by butanol extraction, followed by gas chromatography, as described by Varma et al. (*12*). A calibration curve of DCW vs optical density at 660 nm was used to measure yeast cell concentration in the broth.

RESULTS AND DISCUSSION

Investigations were carried out in shake flasks using various nonosmophilic and osmophilic yeasts in the absence and presence of sodium sulfite or sodium carbonate as a steering agent. The strains (in all, twenty-four) tested belonged to the genera *Saccharomyces*, *Torulopsis*, and *Pichia*. The yields were calculated as grams of glycerol, ethanol, and biomass per 100 g of glucose consumed. It was observed that glycerol yields varied widely with the nature of yeast strain and were appreciably influenced by the presence of sodium sulfite or sodium carbonate. Also, the glucose consumption rates (correspondingly, fermentation times) were positively influenced by the presence of the steering agent for all yeast strains. In the case of nonosmophilic yeasts, the presence of sulfite gave higher glycerol yields than the sodium carbonate. The situation is reversed in the case of some osmophilic yeasts. The overall ranges of pH at harvest and yields of glycerol, ethanol, and biomass in the absence and presence of the steering agent are given in Table 1. It shows that the pH of the fermentation medium appears to be strongly correlated with glycerol yields and fermentation times. On the whole, relatively high glycerol yields, reduced fermentation times, and low ethanol and biomass yields were obtained with osmophilic yeasts in the presence of sodium sulfite or sodium carbonate. An important advantage of using

TABLE 1
Range of pH, Glycerol, Ethanol, and Biomass Yields[a]

Type of yeast strain	Sodium sulfite or sodium carbonate, based on glucose[b]	pH range at harvest	Fermentation time, h	Yields based on glucose consumed, %		
				Glycerol	Ethanol	Biomass (DCW)
Nonosmophilic	Nil	2.2–2.8	120	3.5–7.2	16.5–24	3.6–6.0
	40% sodium sulfite	6.8–7.4	120	16–21	8.4–12	5.8–11.8
	10% sodium carbonate	5.0–6.8	96–120	11–20	14.1–25	8.0–10.5
Osmophilic	Nil	2.4–3.2	24–48	7–18	11–27	2.5–5.8
	40% sodium sulfite	7.2–8.1	24–72	16.4–34	10–19.2	2.2–5.4
	10% sodium carbonate	5.6–8.2	24–72	8.5–40	9.2–26	1.9–5.6

[a]In the absence and presence of sodium sulfite/sodium carbonate for various nonosmophilic and osmophilic yeasts.
[b]Initial glucose concentration, 100 g/L.

osmophilic yeasts is that higher initial substrate concentrations could be used to get correspondingly higher glycerol contents in the product broth, which is important from the recovery point of view (*13*). Furthermore, at high initial glucose concentrations, the chances of the growth of contaminants are likely to be reduced. Therefore, further studies were continued with an osmophilic yeast, *P. farinosa* (ATCC 20210), which gave the highest glycerol yields both in the absence and presence of sodium sulfite or sodium carbonate.

Dose and Mode of Addition of Sodium Carbonate

The form, dose, and mode of addition of sodium carbonate to the medium have an appreciable effect on the glycerol yields (*14*). The effect of different doses of sodium carbonate and various policies of its addition, involving equal or unequal portions at equal or unequal intervals of time in solution, as well as in solid form (fine powder), was investigated and reported in a separate communication (*10*). It was found that the optimum dose of sodium carbonate was 10% (based on glucose), and its addition in solid form in five portions (15, 15, 27.5, 27.5, and 15%) at different intervals of time (4, 7, 9, 12, and 24 h after the start of fermentation) was found to keep the media pH and, consequently, the cell growth at an optimum level to give higher glycerol yields. Further investigations were carried out under this policy of sodium carbonate addition.

Dose and Mode of Addition of Sodium Sulfite

The presence of sodium sulfite in the medium has a positive effect on the glycerol yields. The effect of the dosage of sodium sulfite on the fermentation is given in Fig. 1. It shows that glycerol yields increase from 11 to 37%, with an increase in sulfite dose from 0 to 40% (based on glucose) in a fairly linear manner. These results are in accordance with the observations of Freeman and Donald (*14*), who postulated that at a high sulfite concentration, when the formation of acetaldehyde-bisulfite complex is favored, its subsequent dissociation is suppressed, which in turn favors glycerol formation rather than ethanol. The corresponding ethanol and biomass yields obtained varied from 15 to 7.2% and 2.5 to 0.58%, respectively. Along with the increase in the glycerol yields obtained, the time for completion of fermentation was also reduced from 192 to 96 h, as a result of higher glucose consumption rates (increased from 1.6 to 3.2 g/L/h).

Unlike sodium carbonate, the form and mode of addition of sodium sulfite to the media was found to be less critical. It was sufficient to add the solid sodium sulfite in two equal portions, one at a stage when active fermentation had begun, i.e., 4–6 h after the start and the other 6 h after the first addition.

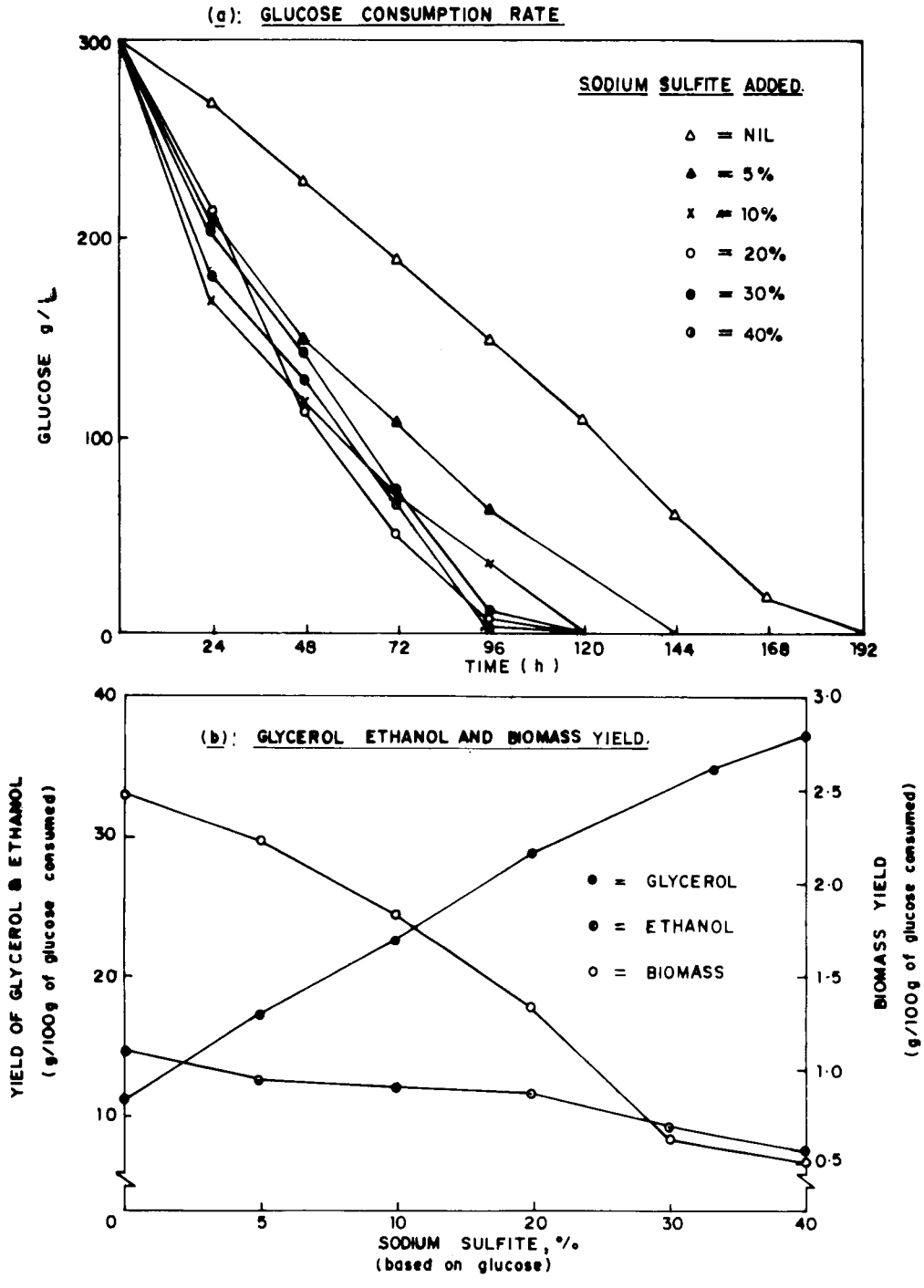

Fig. 1. Effect of dosage of sodium sulfite on glycerol fermentation.

The disadvantage of using sodium sulfite is that it is toxic to the organism beyond a particular value. Further, to achieve 37% glycerol yield, 40% sodium sulfite should be used, whereas to get the same glycerol yield, only 10% sodium carbonate is required, and it is known that in-

creased amounts of salts complicate the recovery process. Therefore, further studies were continued with sodium carbonate.

Size and Age of Inoculum

Although the importance of the inoculum in determining the productivity of industrial fermentations has long been recognized, quantitative investigations have been few (*15*). The inoculum size and age significantly influence the rates of fermentation, as well as the product yields, and the results of the investigations by varying these parameters are shown in Table 2 and Fig. 2. From Table 2 it can be concluded that with 5 and 10% (v/v) inoculum, the sugar utilization was far from complete, but with 15% inoculum the sugar utilization rate was 0.125 g/L/h and the glycerol yield was 40%. A 20% inoculum (this corresponds to 0.1 g DCW/100 mL medium) resulted in a slight reduction of glycerol to 35%, and the glucose utilization rate was slightly improved to 0.140 g/L/h. In all these cases, fermentation completion times exceeded 200 h. Since it is important to reduce the time of fermentation, addition of even higher inoculum was investigated by adding centrifuged cells to obtain different starting concentrations of yeast cells in the medium. For these studies, inoculum values were expressed as g DCW/100 mL medium, and the results are shown in Fig. 2. It was observed that an increase in inoculum from 0.31 to 0.94% reduced the fermentation time from 144 to 96 h and increased the glucose consumption rate from 0.194 to 0.312 g/L/h. However, the glycerol yields decreased from 43 to 30%, and the corresponding ethanol and biomass yields increased from 9 to 14.5% and 0.45 to 1.7%, respectively. Possibly, increased inoculum size results in a greater oxygen requirement, which cannot be met on the shaker because of limited oxygen availability. Under such oxygen-starved conditions, the metabolic pathway is shifted toward ethanol formation, thus reducing glycerol yields. Since decreased inoculum size had a positive effect on glycerol yield and a negative effect on fermentation rate, a compromise value of 0.63 g DCW/100 mL medium was selected for further studies.

TABLE 2
Effect of Inoculum Size (v/v) on Glycerol and Ethanol Yields Under Alkaline Conditions

Inoculum size, %, v/v	Glucose consumed,[a] g/L	Fermentation completion time, h	Yields based on glucose consumed, %	
			Glycerol	Ethanol
5	60	240	33	4
10	80	240	37	4
15	300	240	40	8
20	300	216	35	10

[a]Initial glucose concentration, 300 g/L.

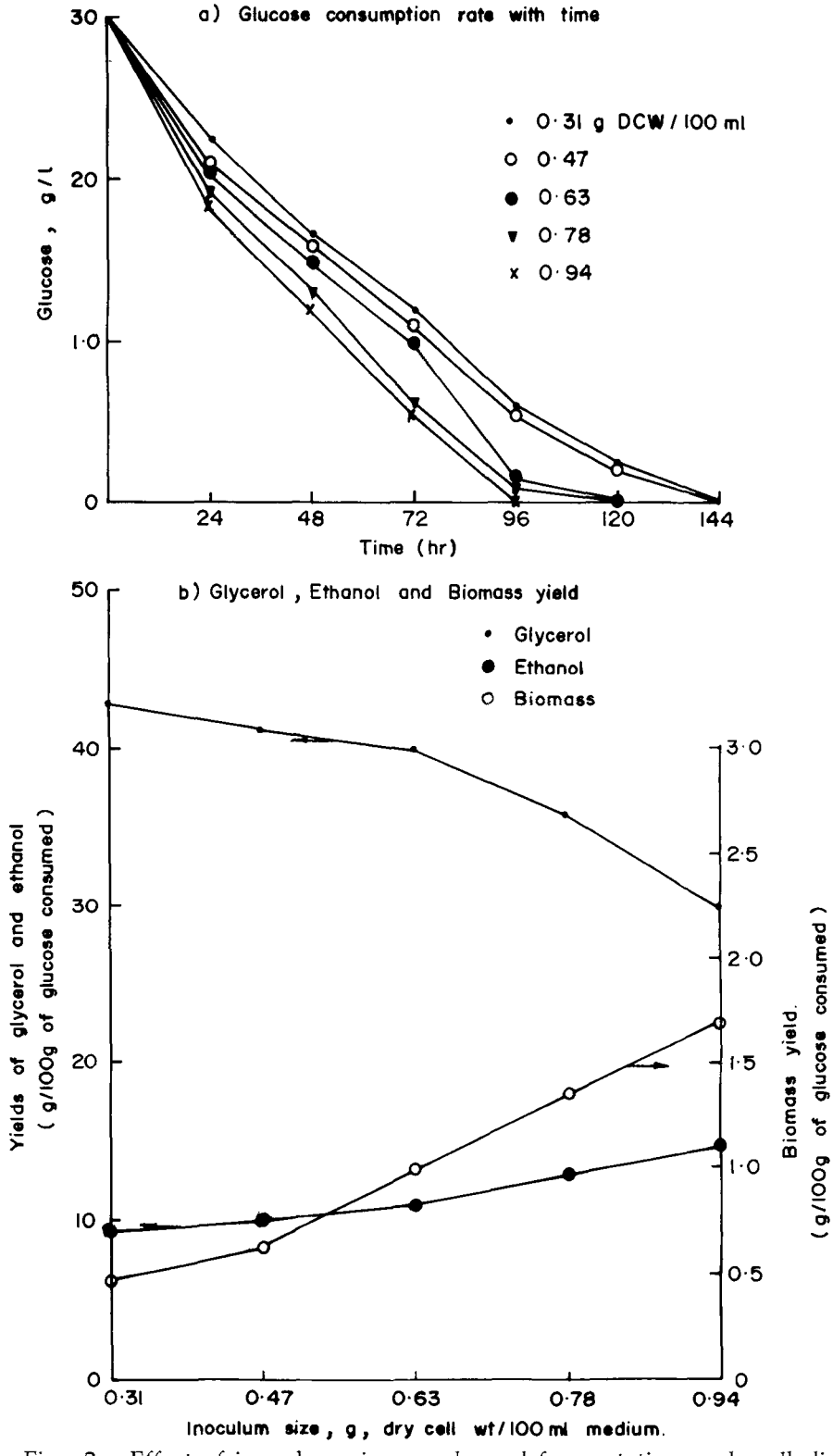

Fig. 2. Effect of inoculum size on glycerol fermentation under alkaline conditions.

This gave reasonably high glycerol yields (40%) within a 120-h period, at a glucose consumption rate of 0.25 g/L/h.

The age of inoculum had a limited effect on the rate of fermentation as well as on the product yields. Young cultures (18–36 h) took 120 h for completion of fermentation at a glucose consumption rate of 0.25 g/L/h, whereas the older cultures (48–96 h) took 96 h at a glucose consumption rate of 0.312 g/L/h. However, glycerol yields based on the glucose consumed are not very different and appear to be slightly higher in the older than in the younger cultures.

Effect of Aeration

Glycerol production by osmophilic yeasts is essentially an aerobic process. In the shake flask, therefore, it depends critically upon the rate of oxygen transferred from the gas head space into the liquid medium, which is partly controlled by the ratio, R, of the volume of the medium to the total capacity of the flask. Therefore, in order to investigate the effect of degree of aeration, this ratio was varied by changing the volume of the medium (20–200 mL) held in a 500-mL shake flask (R varying from 0.04 to 0.40). The change in the ratio R caused a variation in the interfacial area for oxygen transfer. As seen in Fig. 3, the degree of aeration had a marked influence on the rate of fermentation as well as on the product yields. As the ratio R increased from 0.04 to 0.40, the glycerol yields decreased from 50 to 28%. Further, the sugar utilization rate was also decreased from 0.312 to 0.145 g/L/h, thereby prolonging the fermentation time from 96 to 240 h. Corresponding ethanol yields increased from 2.5 to 18%. Similar results were reported by Onishi et al. (*16*), but in their studies the sugar utilization (with the R range from 0.24 to 0.40) was poor, whereas in our studies the sugar utilization was total, probably because of the high inoculum and alkaline conditions used. The biomass yield showed a maximum at $R = 0.16$. It increased from 0.6 to 1.2 when R was raised from 0.04 to 0.16 and decreased from 1.2 to 0.6 when R was increased from 0.16 to 0.40. This maximum in biomass production with varying R has been reproducible. The following reason is attributed to this observation. As the volume of the medium increases, the size of inoculum (0.63 g DCW/100 mL medium) increases proportionately, requiring greater amounts of oxygen. Up to a value of $R = 0.16$, this increased oxygen requirement is met easily. However, beyond this value of R, the increased requirement of oxygen by the cells cannot be met adequately, which in turn favors ethanol formation and low glycerol and biomass yields.

Effect of Glucose Concentration

The substrate concentration is found to influence glycerol formation in most fermentations employing osmophilic yeasts (*17*). Onishi et al. (*6*), working with *Pichia miso*, observed that the yields of glycerol rose from

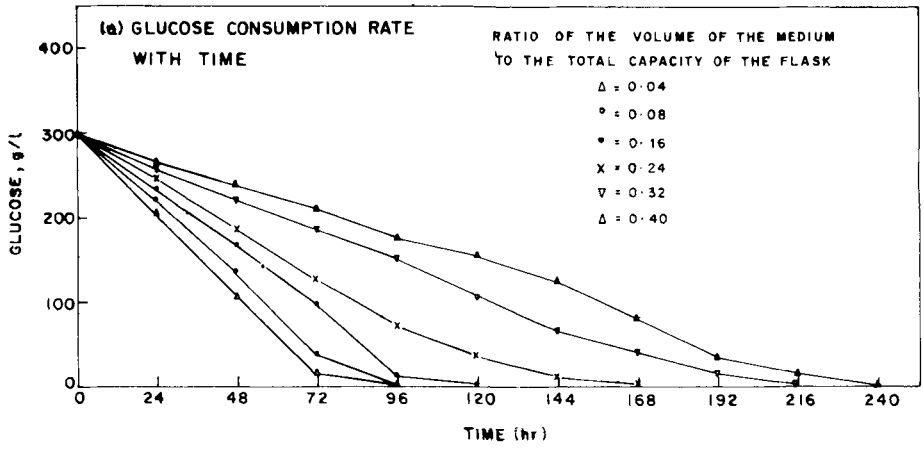

Fig. 3a. Effect of aeration on glycerol fermentation under alkaline conditions. Glucose consumption rate with time.

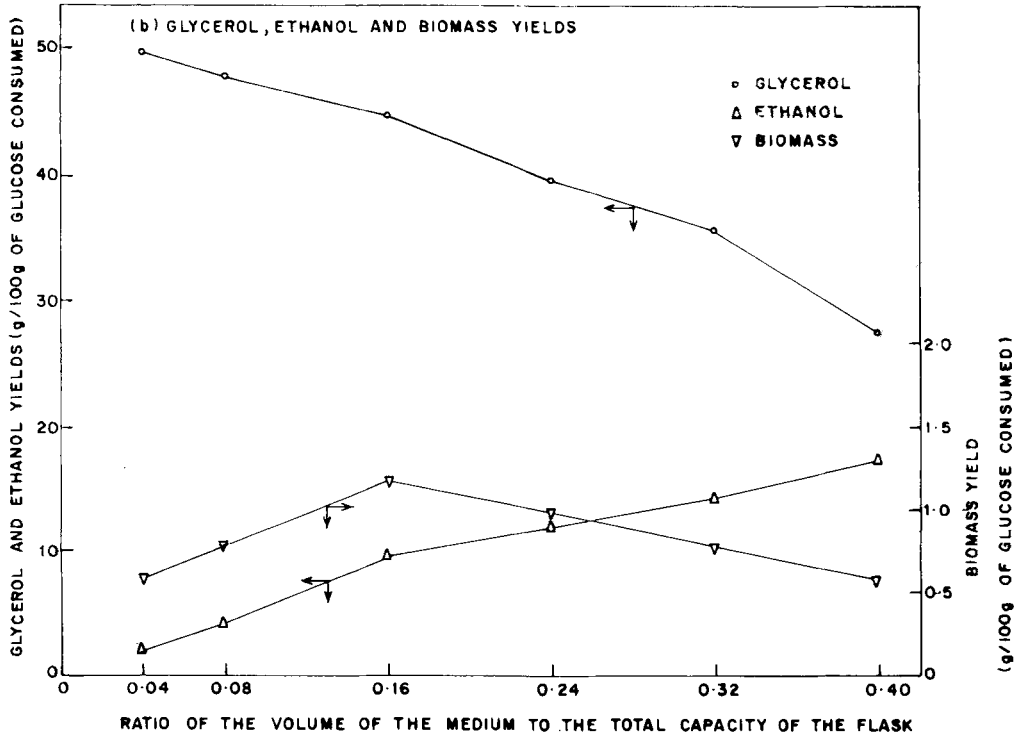

Fig. 3b. Effect of aeration on glycerol fermentation under alkaline conditions. Glycerol, ethanol, and biomass yields.

18 to 43% as the initial glucose concentration was raised from 9 to 29%. Up to this value, nearly all the sugar in the medium was utilized, but when the starting glucose concentration was raised to 48%, the glycerol yield fell to 33%, and the percentage of sugar utilized decreased to 15%. The fermentation times were also high in all the glucose concentrations. Our results (Fig. 4) on the effect of initial glucose concentration (10–50%) on *P. farinosa* also followed a similar pattern. Glucose utilization, how-

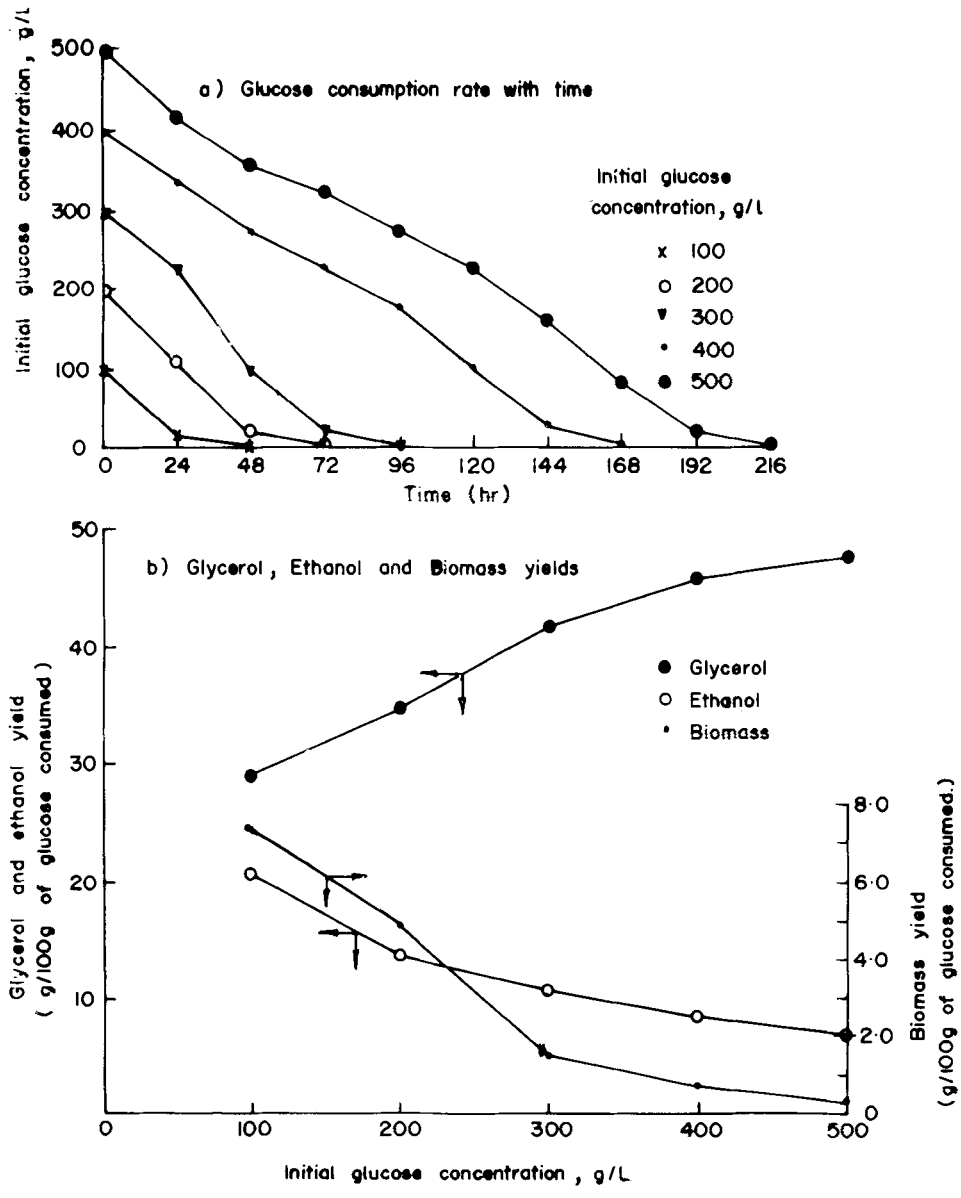

Fig. 4. Effect of initial glucose concentration on glycerol fermentation under alkaline conditions.

ever, was complete in all the cases. From Fig. 4 it is observed that higher initial glucose concentrations gave increased glycerol yields, but this effect tapered off beyond 40%. Increasing the glucose concentration from 10 to 50% increased the glycerol yields from 29 to 48%, and the corresponding glucose utilization rates decreased from 0.41 to 0.23 g/L/h, thereby prolonging the fermentation time from 36 to 216 h. Respective ethanol and biomass yields decreased from 21 to 7% and 7.5 to 0.3%, respectively. Thus, at a lower substrate concentration, the rate of fermentation was rapid, and the glycerol yields were suppressed, whereas at higher substrate concentrations, the glycerol yields were higher, but the

glucose utilization rate was slow, thereby extending the fermentation time. For further studies, a value of 30% initial glucose concentration was chosen since it gave reasonably good glycerol yields in relatively short times of fermentation.

Effect of Carbon Sources

The results of the investigation on the effect of various sugar substrates on the growth of *P. farinosa* show that cell growth is good when the carbon source is essentially glucose, fructose, glycerol, or mannitol. Xylose was tested, keeping in view its availability in the form of xylans in hemicellulosic substrates, and it was found to support reasonably good growth. In view of the necessity of using inexpensive and abundantly available carbon sources, sugar cane molasses, as well as hydrolysates of bagasse, rice straw, and starch, were tried. When molasses was used as the carbon source, there was little growth. This may be a result of the fact that invertase may be inactive in *P. farinosa* under alkaline conditions. Cellulose hydrolysates of rice straw and bagasse worked as good substrates for growth. However, glycerol yields were relatively low, at 15 and 12%, respectively. But starch hydrolysate worked as an excellent substrate for growth, as well as glycerol yields (38%), which were equivalent to that of glucose and fructose. Thus, these results indicate that there are good prospects for using hydrolysates of starch, bagasse, rice straw, and the like, as potential commercial substrates.

There is absolutely no utilization of arabinose, lactose, and maltose, and sucrose is utilized poorly, with no glycerol production.

Effect of Nitrogen Sources

Yeast Extract and Urea

Yeast extract, being an autolyzed form of yeasts, serves as a rich nitrogen source and also supplies other growth promoting factors. Hajny et al. (*18*) observed that a high concentration of yeast extract in the medium brings about a rapid fermentation and an increase in ethanol production, with a corresponding decrease in glycerol yields. Similar results were obtained in our studies, and it was observed that the presence of nitrogen either in the form of yeast extract or urea has a negative effect on the glycerol yields, but fermentation times are reduced. However, its total absence in the medium resulted in poor fermentation rates. On the contrary, a medium with high nitrogen content (4% yeast extract or 2% urea) gave rapid fermentations but low glycerol yields. Based on these results, it was found that a concentration of yeast extract and urea (0.1% and 0.05%, respectively) was optimum, which gives relatively high glycerol yields as well as faster fermentations. This corresponds to a nitrogen level of 0.03% in the medium.

Other Nitrogen Sources

The potential of a number of other useful nitrogen sources, such as peptone, urea, corn steep liquor (CSL), phosphates, sulfates and nitrates of ammonia, and so on, was examined, and the results are shown in Table 3. It is apparent from the table that the inorganic nitrogen sources, such as ammonium sulfate, nitrate, and chloride, as the sole nitrogen source gave good glycerol yields in contrast to the observations of Onishi (*6*) using *P. miso*. This may result from the regulation of pH by addition of sodium carbonate in our studies, whereas in the experiments of Onishi (*6*), the pH decreased so sharply that the fermentation could not continue unless it was buffered. Of the complex nitrogen sources, polypeptone was found to give maximum glycerol yields. Cheaper nitrogen sources, such as CSL and soybean meal, also gave equally good results, indicating that these cheaper materials can be used as commercial nitrogen sources. In the control set containing no nitrogen, sugar utilization was very slow at 0.15 g/L/h, but the glycerol yields were quite high (50%).

Effect of C/N Ratio

The effect of the C/N ratio on product yields and the rate of fermentation are presented in Table 4. It was observed that a C/N ratio of 400:1 gave the highest glycerol yields, and even the fermentation times are not affected adversely. An increase in the C/N ratio from 10 to 400 increased the glycerol yields from 26 to 45%, the fermentation times from 96 to 144 h, and the glucose utilization rates were decreased from 0.312 to 0.21 g/L/h. Corresponding ethanol and biomass yields fell from 19 to 11% and 4 to 1.3%, respectively. Even though increasing the C/N ratio beyond 400

TABLE 3
Effect of Various Nitrogen Sources on Glycerol, Ethanol, and Biomass Yields Under Alkaline Conditions[a]

Nitrogen source	Fermentation completion time, h	Yields based on glucose consumed, %		
		Glycerol	Ethanol	Biomass (DCW)
L-Glutamic acid	144	38	9	1.8
Ammonium lactate	144	35	10	1.7
Ammonium sulfate	144	36	10	1.8
Ammonium chloride	144	40	9	1.8
Beef extract	144	40	11	1.4
Yeast extract	120	39	12	1.5
Polypeptone	144	45	11	1.6
Soybean meal	168	37	14	1.9
Corn steep liquor	144	41	13	1.8
Urea	144	42	11	1.3
Nil	240	50	8	0.05

[a]Initial glucose concentration, 300g/L, nitrogen supplied 0.3 g/L.

TABLE 4
Effect of C/N Ratio on Glycerol, Ethanol, and Biomass Yields Under Alkaline Conditions

C/N ratio	Fermentation completion time, h	Yields based on glucose consumed, %		
		Glycerol	Ethanol	Biomass
10	96	26	19	4.0
25	96	30	17	3.2
50	120	34	15	2.5
100	120	38	12	1.9
200	120	41	12	1.6
400	<144	45	11	1.3

might give slightly higher glycerol yields, the time of fermentation would be unduly prolonged.

Effect of Temperature

Investigations were carried out on the effect of temperature on the growth of *P. farinosa* and on the product yields, and the results are shown in Table 5. The table shows that the glucose utilization rate was increased from 0.38 to 0.80 g/L/h as the temperature was raised from 25 to 40°C. However, the glucose utilization rate fell to 0.41 g/L/h when the temperature was further raised to 45°C. The corresponding biomass yields also decreased from 2.6 to 0.6%. The change in the glycerol yields was small in the temperature range of 30–40°C, but at 45°C the glycerol yields increased to 44%. This probably resulted from the higher availability of dissolved oxygen since the cell growth at this temperature is minimal. However, 45°C is not conducive to growth, and fermentations became prolonged. From these investigations, 40°C appears to be the most suitable temperature of fermentation. This is of significance in improving the overall productivity. Further, the advantages of a higher temperature operation are the ease of cooling the fermentor in an industrial situation and the reduced need for aseptic conditions for operation.

TABLE 5
Effect of Temperature on the Product Yields of *P. farinosa* Under Alkaline Conditions[a]

Temperature, °C	Yields based on glucose consumed, %			Glucose utilization rate, g/L/h
	Glycerol	Ethanol	Biomass	
25	28	24	2.6	0.38
30	36	20	1.9	0.44
35	38	18	1.6	0.66
40	37	14	1.0	0.80
45	44	8	0.6	0.41

[a]Initial glucose concentration 100 g/L.

Cell Recycle

In order to minimize the lag phase and to get faster rates, a large inoculum has been found to be useful (*19*). The time and effort in developing the inoculum can be saved if cells from one batch of fermentation could be reused in the next batch. The feasibility of such a recycle under alkaline conditions was investigated in the following way. After harvesting a batch, the cells were centrifuged at 4°C for 15 min at 2200*g* and washed twice with saline. From this, a sufficient cell mass (equivalent to the inoculum of the first batch) was used as an inoculum for the next batch of fermentation. No particular care was taken to maintain aseptic conditions during the cell recycle, except that the cells were added into the medium under flame. This operation was repeated 10 times, and the results are shown in Table 6. The glycerol yields and fermentation times were almost the same in all the batches. There was no trend of decline in the yields, indicating that perhaps many more reuses were possible. This is important from the economic point of view since it saves time, labor, and money involved in the preparation of inoculum (*20*).

Nonsterile Operation

In fermentation processes, particular attention should be paid to sterilization and to operations to secure freedom from contamination. In our studies, the glucose solutions, salt solutions, and solutions containing nitrogen are sterilized in an autoclave at 15 psi, for 20 min. These are sterilized separately to avoid caramelization and deterioration of the medium and are mixed together under flame after cooling. The inoculum is also added under aseptic conditions, and the fermentation is then carried out under strict asepsis. Since the medium sterilization and maintenance of sterilization during fermentation involves considerable expenditure, investigations were carried out for studying the feasibility of fermentation under nonsterile conditions and the media being not steril-

TABLE 6
Yields of Glycerol with Reuse of Yeast Cells Under Alkaline Conditions[a]

Reuse number	Yield of glycerol, %
0	40
1	41
2	38
3	37
4	39
5	41
6	35
7	38
8	38
9	40

[a]Initial glucose concentration 300 g/L; fermentation time, 120 h.

ized. The results show that there is no difference in the glycerol yields and the fermentation times compared to that of the fermentations under sterile conditions. This may be a result of the following factors:

(1) High initial glucose concentrations (30%).
(2) High inoculum used.
(3) Alkaline pH conditions.

CONCLUSIONS

Various nonosmophilic and osmophilic yeasts were screened for their ability to produce glycerol in the absence and presence of sodium sulfite or sodium carbonate. Of these it was found that an osmophilic yeast *P. farinosa* was promising. Optimization of media and process conditions for high glycerol yields and reduced fermentation times in shake flasks have been carried out. Although alkaline fermentation has been reported to give good glycerol yields using nonosmophilic yeasts, it was not tried previously with osmophilic yeasts. A systematic study was carried out using *P. farinosa* under alkaline conditions, which resulted in higher glycerol yields, coupled with faster fermentations.

The age of inoculum had little influence on the glycerol yields, an increased inoculum size resulted in faster fermentations and lower glycerol yields. Aeration was found to be critical for enhanced glycerol yields. It was shown that it was possible to reuse yeast cells without affecting the fermentation times and glycerol yields. Cheaper nitrogen sources, such as CSL and soybean meal, and a carbon source such as starch hydrolysate were found to give good glycerol yields. A reduction in nitrogen in the medium increases glycerol yields, but fermentations took a longer time. Operations at higher temperatures were found to be beneficial up to about 40°C, and under these experimental conditions, strict sterile conditions are not essential.

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