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Glycerol Production by Fermentation

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ABSTRACT

In the context of rapidly depleting petroleum reserves, production of chemicals from renewable carbohydrate-rich resources has assumed importance and a particularly good candidate process is the production of glycerol by fermentation. Of the various osmophilic and non-osmophilic yeasts tested for glycerol yields, two osmophilic yeasts were selected for further studies. The effect of sodium chloride concentration, inoculum size, and nitrogen content of the medium have been investigated. Centrifugation and recycling of cells does not appreciably affect the glycerol yield and fermentation times.

Index Entries: Osmophilic yeasts, in glycerol fermentation; non-osmophilic yeasts in glycerol fermentation; glycerol, production by fermentation; ethanol, cell recycle; glucose utilization; aeration, in glycerol fermentation; yeasts, in glycerol fermentation; fermentation, glycerol production by;

INTRODUCTION

Interest in glycerol production by fermentation was started when Neuberg et al. (*1*) proposed their mechanism for the formation of glycerol in the presence of sulfites. Subsequently, it led to a commercial-scale operation using yeasts, particularly during the World War I. Later, many studies were conducted by changing the techniques and microorganisms. Eoff et al. (2) used alkaline conditions with sodium carbonate, and

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various other alkaline agents were used for the production of glycerol on a pilot-scale. Further studies showed that not only yeasts, but also other microorganisms, such as *Mucor, Streptococci, Aspergilli, Pencillia,* etc., produce glycerol and/or other polyalcohols. Neish et al. (*3*) tried bacteria as a potential industrial source of glycerol; however, only poor yields were achieved. Craigie and MacLachan (*4*) reported that a species of algae, *Dunaliella,* forms glycerol as a byproduct of photosynthesis and recently more reports (*5,6*) have appeared on this technique as a result of an active interest in the production of glycerol from renewable resources. The entire scenario was changed when Onishi (*7*) and Spencer et al. (*8*) showed that glycerol could be produced in large amounts in the absence of sulfites or alkaline agents with osmophilic yeasts. Although these investigations were promising, they did not lead to the commercial production of glycerol by fermentation, partly because a much cheaper method was available for the production of glycerol from the then abundantly available petroleum byproducts, and partly because of the difficulties in recovering the product from the beer. However, since petroleum reserves are now seen to be depleting fast, interest in the production of chemicals from renewable carbohydrate-rich resources has been revived, and a particularly good candidate is the production of glycerol by fermentation. Further, with the advent of recent techniques such as reverse osmosis, ultrafiltration, ion-exchange, ion exclusion, etc, it may be simpler to recover the product. In the present study, conditions favoring the production of glycerol from glucose have been investigated in shake flask experiments using various osmophilic and non-osmophilic yeast cultures and the effect of various operational parameters on the yield as well as productivities has been reported.

MATERIALS AND METHODS

Cultures

The yeast cultures were obtained from National Collection of Industrial Micoorganisms (NCIM, Poona) and American Type Culture Collection (ATCC, USA) and the specific strain numbers are listed in Table 1. All the cultures were maintained on MGYP–agar slants.

Shake Flask Experiments

A loopful of culture from the stock agar slant 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 and was grown on the shaker for 24 h. The cells were then centrifuged at 4°C for 15 min at 4000 rpm.

Wet cells corresponding to 0.5 g dry wt were suspended in 5 mL of the medium and inoculated into a 500 mL shake flask containing 80 mL

TABLE 1
Yields of Glycerol, Ethanol, and Biomass with Different Yeast Strains in Shake Flask Experiments[a]

Strain number	Nature of yeast strain	Fermentation time, h	Yields based on glucose consumed		
			Glycerol, %	Ethanol, %	Biomass (DCW), %
NCIM 3193	*S. cerevisiae;* non-osmophilic	120	5.0	18.0	3.78
NCIM 3194	*S. cerevisiae;* non-osmophilic	120	7.0	19.0	5.5
NCIM 3195	*S. cerevisiae;* non-osmophilic	120	6.0	20.0	5.8
NCIM 3288	*S. cerevisiae;* non-osmophilic	120	3.5	20.0	5.7
NCIM 3095	*S. cerevisiae;* non-osmophilic	120	5.0	24.0	6.0
NCIM 3263	*S. rouxii;* osmophilic	24	7.5	26.0	4.38
NCIM 3264	*S. rouxii;* osmophilic	24	7.0	27.0	3.7
NCIM 3376	*S. rouxii;* osmophilic	48	9.0	18.0	5.8
NCIM 3291	*S. rouxii;* osmophilic	48	8.0	12.0	4.1
ATCC 13356	*S. rouxii* osmophilic	48	9.5	16.0	2.52
ATCC 20210	*Pichia farinosa,* osmophilic	48	18.0	14.0	5.0

[a]Initial glucose concentration, 10%. NCIM, National Collection of Industrial Microorganisms, NCL, Pune, India. ATCC, American-Type Culture Collection, USA.

of media consisting of 10% glucose; 0.1% yeast extract; 0.05% urea; 0.1% KH_2PO_4; 0.05% $MgSO_4 \cdot 7H_2O$; and 0.01% $CaCl_2 \cdot 2H_2O$. Whenever the medium composition was different, it has been indicated. The flasks after inoculation were incubated at 30°C on a rotary shaker at 180 rpm. At specified time intervals, 2–3 mL of sample were drawn aseptically and, after removal of the yeast cells by centrifugation, the clear fermented broth was subjected to analysis for residual glucose, glycerol, and ethanol.

Analytical Procedures

Glucose concentration was measured enzymatically using a glucose analyzer (Yellow Springs Instruments, USA) with glucose oxidase enzyme. Glycerol concentration was measured by the periodate oxidation method of Neish (*9*). Ethanol was estimated by gas chromatography using 80–100 mesh Chromosorb 101 column with an oven temperature of 150°C; The injector and TCD temperature was 210°C and the H_2 carrier gas flow rate was 30 mL/min; isopropanol was used as an external standard. For the measurement of cell growth the turbidity was measured in a UV–visible spectrophotometer (Shimadzu model 240) at 660 nm against an uninoculated blank and the cell concentration was read out from the calibration graph of dry cell weight (DCW) vs optical density.

RESULTS

Various osmophilic and non-osmophilic yeast cultures tested gave yields of glycerol varying widely with the nature of yeast strain, as seen in Table 1. The yields were calculated as grams of glycerol, ethanol, and biomass per 100 g glucose. Non-osmophilic yeast cultures gave yields of glycerol, ethanol, and biomass (dry cell weight, DCW) varying from 3.5 to 7%, 24 to 18% and 6 to 3.8%, respectively; corresponding values for osmophilic yeasts were from 7 to 18%, 27 to 12% and 5.8 to 2.5%, respectively. On the whole, osmophilic yeasts gave better glycerol yields than the non-osmophilic yeasts. Glucose utilization rates were also higher with the former, thereby reducing the fermentation times. Further, osmophilic yeasts withstand higher concentrations of sugars, which to some extent minimizes the chances of contamination. Therefore, osmophilic yeasts were used for further studies, and among them, *Saccharomyces rouxii* (ATCC 13356) and *Pichia farinosa* (ATCC 20210) were found to give relatively better yields of glycerol (*see* Table 1) and were selected.

Effect of Sodium Chloride Concentration

In an earlier report Onishi (*10*) reported that increasing salt concentrations (in the range 0–18%) gave better glycerol yields using *S. rouxii*. However, in his studies the fermentation rate was very low, taking more

than 10 d for the completion of reaction. This probably occurred because the amount of inoculum was not quite adequate. In our investigations a higher inoculum was used, leading to reduced fermentation times of the order of 5–7 d. The effect of sodium chloride concentration was studied in the range 0–20%.

Figure 1a shows that as the concentration of sodium chloride increases from 0 to 20% the glucose consumption rate becomes smaller and fermentation completion time increases from 48 to 192 h. The glycerol yields increase from 12 to 39%, the corresponding ethanol and biomass yields decrease from 16 to 2% and 2.28 to 0.22%, respectively. These results are in accordance with the previous report of Onishi (*10*) on the effect of salt concentration on glycerol yields.

The strain *S. rouxii* gave higher glycerol yields only in the presence of salt in high concentrations, which however took an inordinately long time for completion of the fermentation. Further, higher initial glucose concentration would have an adverse effect on the glycerol yield (*11*). In this context the other strain selected, *P. farinosa* (ATCC 20210) was more promising. It was found that with this strain the fermentation was completed within 144 h for a 26% initial glucose concentration giving nearly 20% of the glucose consumed as glycerol. Therefore, further investigations were carried out with *P. farinosa,* using an initial glucose concentration around 26% since higher glycerol yields are obtained with this strain at higher glucose concentrations.

Effect of Inoculum Size

The quantity of the cells taken in the inoculum has an important bearing on the time of fermentation as well as on the product yields. Therefore, the effect of inoculum was investigated by varying the inoculum from 0.312 to 0.937% (g DCW/100 mL of the media) and the results are shown in Figs. 2a and 2b. It is seen that an inoculum increase reduced the fermentation time from 192 to 120 h. However, the glycerol yields also decreased from 21 to 11% and corresponding ethanol and biomass yields increased from 8 to 17.5% and 1.03 to 2.30%, respectively. In general it was observed that lower glycerol yields were always accompanied by higher ethanol and biomass yields. This can be partly attributed to the limitation of oxygen at high biomass concentrations, causing suboptimal aeration levels. As the inoculum size increases, the demand for oxygen by the cells increases proportionately. The oxygen solubility being constant, this increase in oxygen requirement cannot be provided on the shaker and under such oxygen-starved conditions the metabolic pathway is switched toward more ethanol formation. That inadequate aeration levels can trigger anaerobic metabolism and give reduced glycerol and increased ethanol yields has been recognized (*14*). Since decreased inoculum size had a positive effect on glycerol yield and a negative effect on the rate of fermentation, a compromise value of 0.625% was selected for further studies since it gave reasonably high glycerol yields

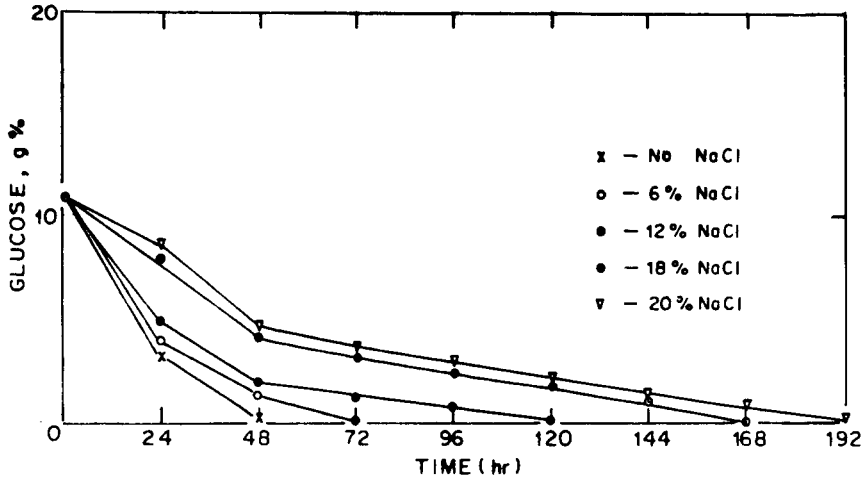

Fig. 1a. Effect of NaCl on glucose consumption rate.

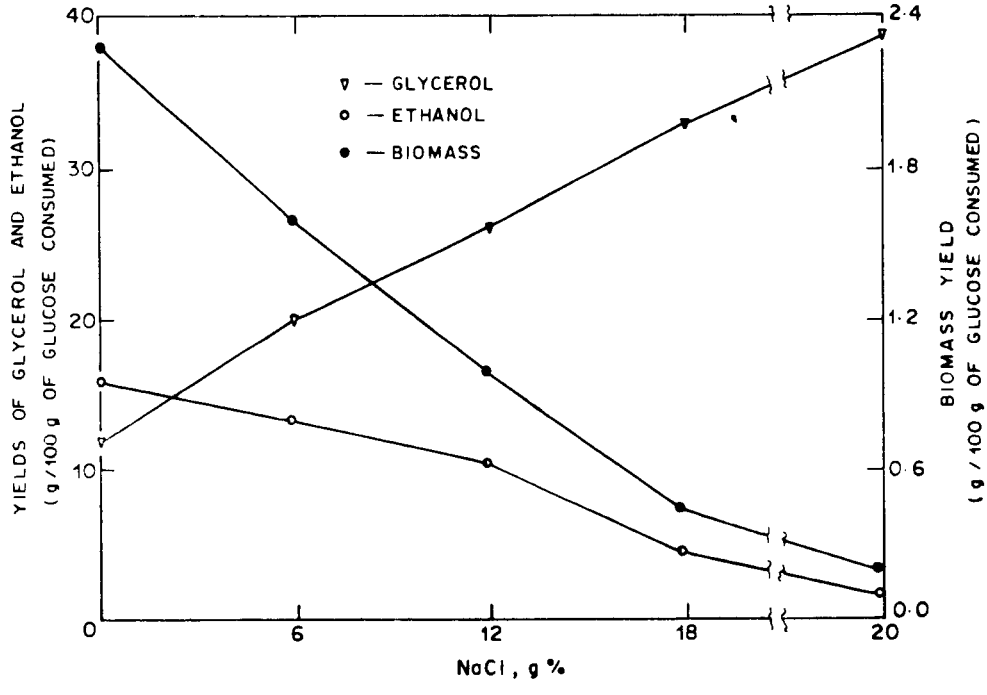

Fig. 1b. Effect of NaCl on glycerol, ethanol, and biomass yields.

within a period of 144 h. This necessitated the centrifugation of cells from the inoculum growth medium, since the direct addition of a large volume of inoculum may result in excessive dilution of the fermentation medium, undesirable pH changes, or carryover of undesired nutrients or

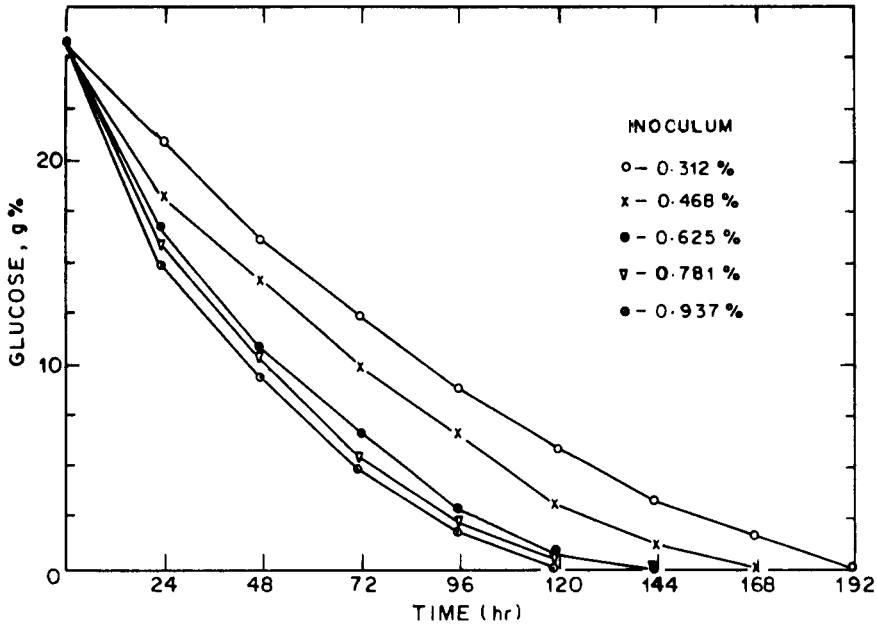

Fig. 2a. Effect of inoculum size on glucose consumption rate.

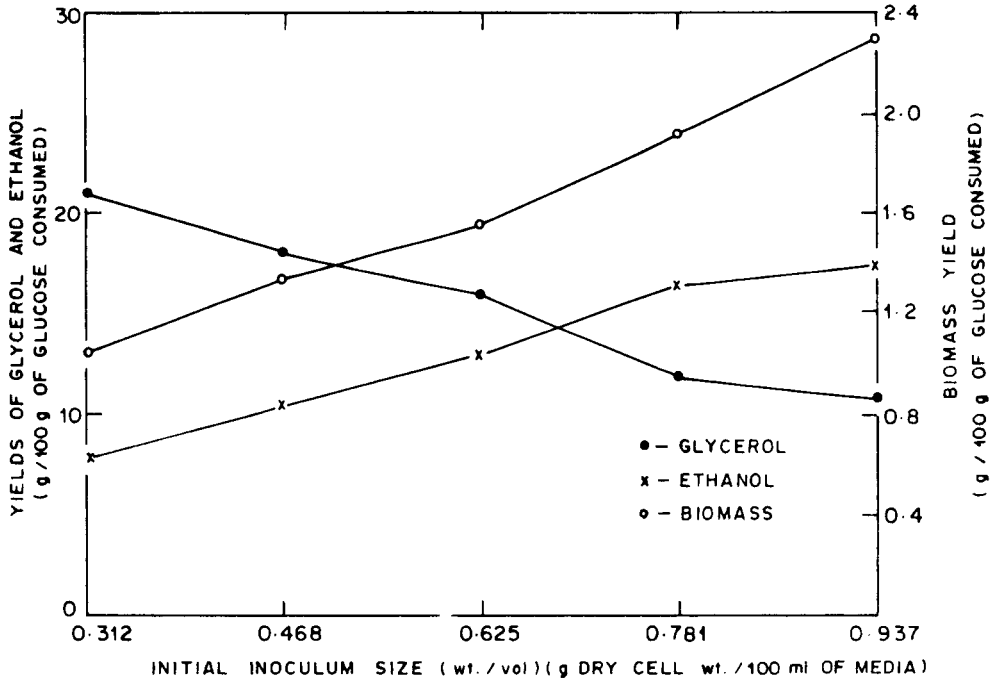

Fig. 2b. Effect of inoculum size on glycerol, ethanol, and biomass yields.

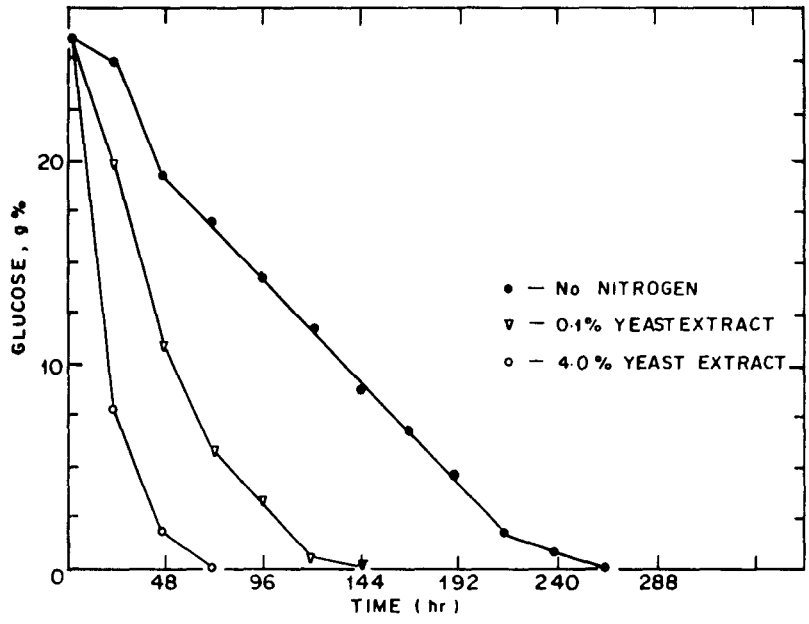

Fig. 3a. Effect of nitrogen content on glucose consumption rate.

metabolic waste products that may sometimes inhibit the fermentation (*12*).

Effect of Nitrogen Source

Nitrogen was found to have a negative effect on the glycerol yields; however, its total absence gave poor fermentation rates. Figure 3b shows that the media with no nitrogen gave the highest glycerol yields (26%), but that the glucose consumption rate was very low, thereby prolonging the fermentation time to 264 h (see Fig. 3a). In contrast, in the media with a high nitrogen content (4% yeast extract) the fermentation was over within 72 h giving a low glycerol yield, 12.30%. Corresponding ethanol and biomass yields are relatively high at 21.15 and 2.79% respectively, whereas in the media with a low nitrogen content (0.1% yeast extract) the reaction was complete within 144 h, giving 19.5% glycerol yield. These results are in accordance with a previous report of Onishi (*13*).

Centrifugation and Recycling of Cells

In order to minimize the lag phase and to get faster fermentations, a large inoculum has been found to be useful. The time and effort in building up this inoculum can be saved if the cells from one batch of fermentation could be reused in the next batch, and this was investigated (using a 10% inital glucose concentration) in the following way. After harvesting a batch, the cells were centrifuged at 4°C for 15 min at 4000 rpm and washed twice with distilled water. From this, a sufficient cell mass

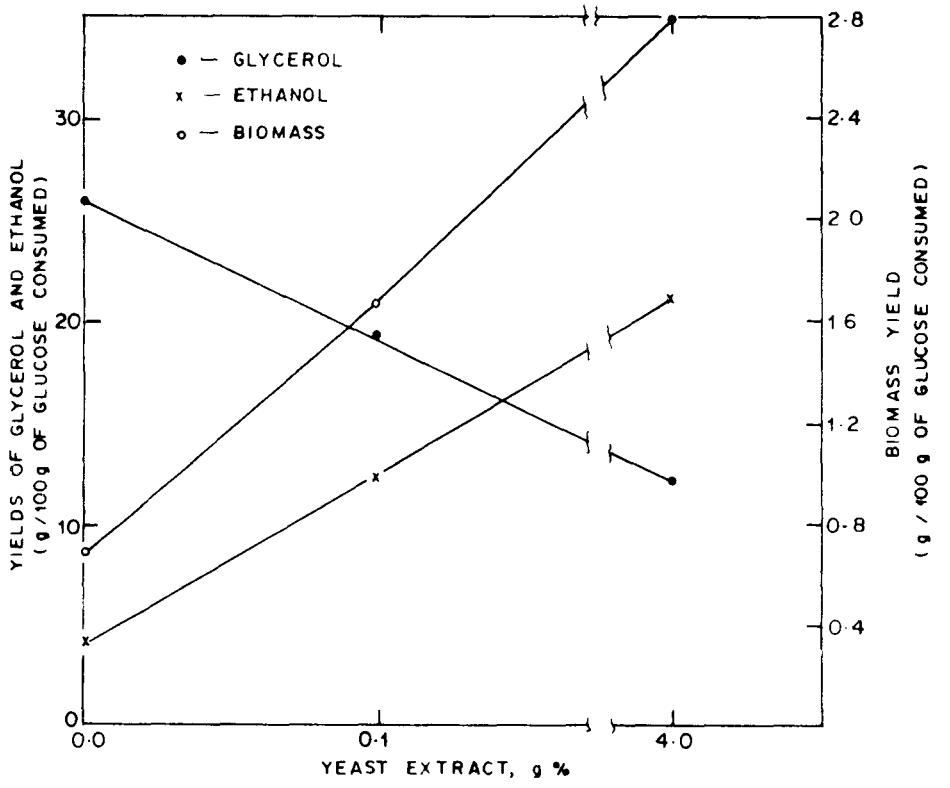

Fig. 3b. Effect of nitrogen content on glycerol, ethanol, and biomass yields.

(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. This operation was repeated five times. The glycerol yields and the fermentation times were the same in all batches, as seen in Table 2.

TABLE 2
Yields of Glycerol with Reuse of Yeast Cells (*P. farinosa*)[a]

Reuse number	Yield of glycerol, %
0 (first batch)	17.0
1	17.0
2	17.5
3	17.3
4	18.0
5	17.7

[a]Initial glucose concentration, 10%; fermentation time, 48 h.

DISCUSSION

Of the various cultures tested for glycerol production, it was found that the glucose consumption rate was higher with osmophilic yeasts. Some osmophilic yeasts gave relatively better glycerol yields than the non-osmophilic yeasts.

With *S. rouxii* (ATCC 13356) under high salt concentrations, glycerol yield was high and ethanol and biomass yields were low. This may happen partly because high concentrations of sodium chloride inhibit cell growth during fermentation. The aeration required for this relatively low cell concentration is likely to be met adequately on the shaker. However, in the media with no sodium chloride, cell growth during fermentation was larger, demanding more oxygen, which cannot be provided on the shaker, and the relatively anaerobic conditions lead towards the ethanol formation. In a previous report, Spencer et al. (*14*) reported that the presence of ethanol was always associated with decreased glycerol yields and that this condition was brought about by suboptimal aeration levels; the optimum aeration in turn varies with the extent of cell growth. This can be clearly seen in Fig. 2a, showing the appreciable effect of inoculum size. A higher inoculum decreased the glycerol yields and increased the ethanol and biomass yields. For the same reason, the glycerol yields at the high salt concentrations reported here are slightly lower than those reported by Onishi (*10*) since a higher inoculum has been used in our studies. Similar observations are made from the effect of nitrogen shown in Figs 3a and 3b. Higher nitrogen content in the media is associated with low glycerol and high ethanol and biomass yields. A higher nitrogen content favors cell growth, which not only consumes a part of glucose but also leads to a limitation of the oxygen concentration, both of which have a direct impact on lowering the glycerol and increasing ethanol yields. Although the total absence of nitrogen gave highest glycerol yields, for practically feasible fermentation times, small amounts of nitrogen will be desirable. However, it may be possible to determine optimum nitrogen concentrations and inoculum sizes that depend on each other. The experiments conducted on the reuse of cells have been found to be promising since five reuses have been possible with no decrease in the yield of glycerol.

The effects of several variables studied in our investigation appear to be strongly related to the dissolved oxygen requirement of the organism during fermentation. Since the aeration levels possible in the shake flask experiments are strictly limited, it would probably be necessary to conduct experiments in a laboratory fermentor with control of variables to obtain more meaningtul conclusions.

CONCLUSION

Of the various cultures tested, two osmophilic yeast strains were found to give promising yields of glycerol. High glycerol yields were obtained in the presence of high sodium chloride concentrations, but it took too long a time for the fermentation to be completed. Nitrogen was found to have an adverse effect on glycerol yields and the medium with no nitrogen gave highest glycerol yields, but resulted in long fermentation times. Five reuses of the cells were possible without affecting either the yields of glycerol or the fermentation times. A combination of the right parameters may give high glycerol yields as well as reduced fermentation times, and further investigations in this direction are in progress.

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