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CULTIVATION OF THE OLEAGINOUS YEAST *CRYPTOCOCCUS CURVATUS* IN A NEW REACTOR WITH IMPROVED MIXING AND MASS TRANSFER CHARACTERISTICS (SURER®)

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SUMMARY

Cultivation and lipid production using the yeast *Cryptococcus curvatus* has proven to be efficient in a fed-batch fermentation using a stirred tank reactor. Scale up of this reactor however results in changing mixing and mass-transfer characteristics. In this paper we report cultivation of the yeast in a new type of reactor (Surer®), which can easily be scaled up. A high cell density (91 gl^{-1}) and a lipid production rate of 0.42 g lipid $l^{-1}h^{-1}$ were obtained.

INTRODUCTION

Oleaginous microorganisms are able to accumulate lipid in the form of intracellular oil droplets. Lipid accumulation specifically occurs under conditions where there is an excess of carbon source and a limiting amount of one of the other nutrients, preferably nitrogen (Granger et al., 1993). *Cryptococcus curvatus* also known as *Candida curvata* or *Apiotrichum curvatum* is an industrial interesting yeast because of its ability to grow on many different carbon sources, including agricultural or food processing wastes (Ykema, 1989). The oil, consisting mainly of triglycerides, can be accumulated up to 60% of the cell dry weight (Ratledge, 1991).

Important parameters determining the price of yeast oil are substrate and fermentation costs. To achieve low fermentation costs a high lipid production rate (g lipid $l^{-1}h^{-1}$) and a high final product concentration are required. In the past, different cultivation modes including batch, fed-batch and continuous fermentations and a broad range of substrates have been investigated. In a recent paper we summarized results obtained in these different fermentations (Meesters et al., 1995). Highest reported lipid production rate (0.995 $gl^{-1}h^{-1}$) was achieved in a partial recycling method by Ykema et al. (1988), using whey permeate as a substrate.

Untill now fermentations were performed in a stirred tank reactor. In case of very high cell densities and a viscous medium, stirring and good mixing becomes a problem in the stirred tank reactor and as a result oxygen transfer is a production rate limiting factor. The stirred tank reactors cannot be scaled up easily due to changing mixing characteristics in bigger tanks. This study describes lipid production by *C. curvatus* using glycerol as a carbon source, in a new type of reactor (Surer®) with high oxygen transfer rates and easy to scale up mixing characteristics.

MATERIAL AND METHODS

Growth media and organism

The lipid accumulating yeast *C. curvatus* ATCC 20509 was used. The yeast was maintained on YPD (2% bacteriological peptone, 1% yeast extract, 2% glucose) agar pH 5.5 and transferred to a fresh plate every four weeks. Fermentation experiments were performed on a minimal growth medium (MM) with glycerol (16 g/l) as sole carbon source. The MM contained per liter demineralized water; KH_2PO_4, 2.7 g; Na_2HPO_4, 0.95 g; $MgSO_4.7H_2O$, 0.2 g; yeast extract, 0.1 g; EDTA, 0.1 g; pH 5.5. After sterilization at 120°C the medium was supplemented with 10 ml per litre of a trace element solution which contained per litre; $CaCl_2.2H_2O$, 4 g; $FeSO_4.7H_2O$, 0.55 g; citric acid.H_2O, 0.52 g; $ZnSO_4.7H_2O$, 0.10 g; $MnSO_4.H_2O$ 0.076 g, 100 µl 36 N H_2SO_4. Nitrogen was added to the medium in the form of base or acid to keep pH at 5.5.

The inoculum for fermentation was precultured overnight on liquid YPG (2% bacteriological peptone, 1% yeast extract, 16 g/l glycerol) medium at pH 5.5 and 30°C.

Fermenter characteristics

In this study a new type of fermenter was used. Figure 1 panel A shows schematically the Surer® reactor, a pumped external loop reactor with two static mixers (Applikon, Schiedam, The Netherlands). The Surer® reactor has a forced flow, resulting in thoroughly mixing with no stagnant zones. The 3 litre fermenter possesses 2 mixers taking care of oxygen transfer, just after the gas inlet. Figure 1 panel B shows in more detail the static mixers, in which the air is reduced to very small bubbles, resulting in an optimal oxygen transfer. The oxygen transfer rate (OTR) can be controlled by the number and type of the static mixers. The mixing time in this fermenter is 5 times the average circulation time. The 3 litre fermenter containing 1.5 litre MM + 24 g glycerol was inoculated with 1.5 litre of an overnight grown culture of *C. curvatus* in YPG medium. Air was supplied at a rate of 0.2 vvm and pumping was performed with a Hitachi HFC-VWS Transistor inverter motor (Vector aandrijftechniek B.V., Rotterdam, The Netherlands) at 20 Hz. Temperature was constantly measured and kept at 28°C using an Applikon ADI1020 control unit. Also the pH was measured on line using the ADI1020 control unit and was kept constant at 5.5. In the first 12 hours of the fermentation pH was controlled by the addition of acid (1 N HCl + 10 g/l NH_4Cl). Later on base was added to remain at pH 5.5. In the first period of biomass production 10% NH_4OH was added as a base, while after 30 hours of cultivation a N-limitation was applied and pH was controlled by the addition of 5 N NaOH. For cooling of the fermenter a cryostat was used. During fermentation 7.5 ml of a substrate mixture containing 87% glycerol (200 ml) / 50x salts (KH_2PO_4 135 g/l, Na_2HPO_4 47.5 g/l, $MgSO_4.7H_2O$ 10 g/l, yeast extract 5 g/l, EDTA 5 g/l, pH 5.5) (100 ml) / trace element solution (see above) (50 ml), was automatically added every 15 minutes. 20 ml Samples were taken every two hours. Foaming was controlled by the addition of a silicon antifoam emulsion SE-2 (Boom B.V., Meppel, The Netherlands).

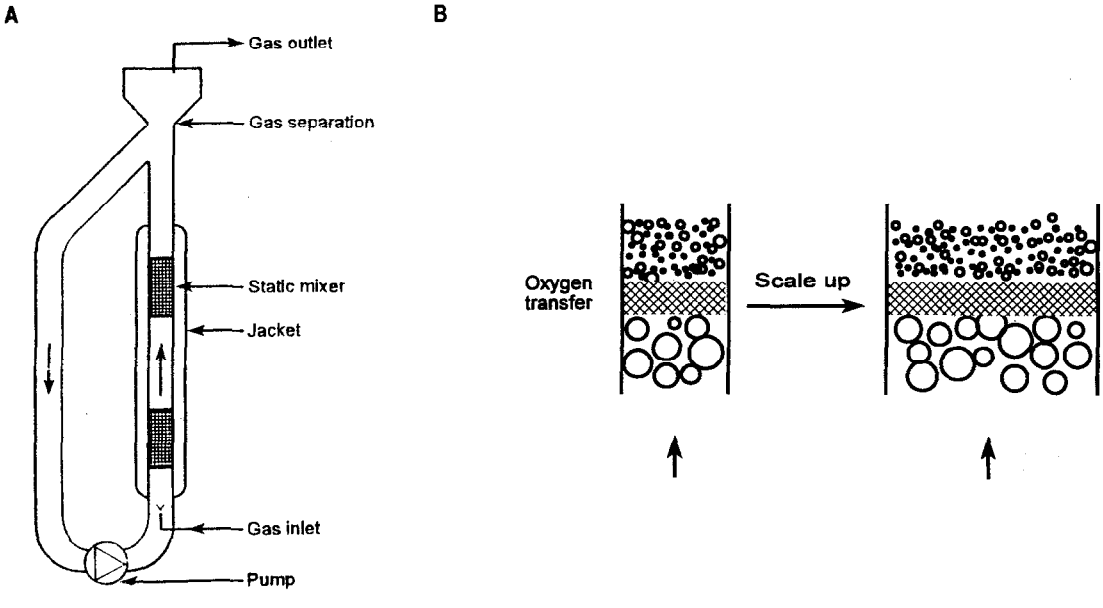

Fig. 1 *Panel A; Schematic representation of the Surer® reactor*
Panel B; Static mixers in more detail

Analytical procedures

Biomass production was followed by measurement of the optical density at 446 nm and cell dry weight was determined every two hours by taking 2 ml of culture broth in preweighed eppendorf tubes, washing twice with demineralized water and freeze dried overnight. Cell dry weight measurements were perfomed in duplicate. The freeze dried cells were used for lipid analysis as described before (Meesters et al., 1995). GC analysis was performed in duplicate from duplicate freeze dried fermenter samples.

RESULTS AND DISCUSSION

Growth of oleaginous yeasts in fermenters has been intensively studied. A fed-batch cultivation mode has proven to be very useful and high cell densities could be achieved with different organisms (Yamauchi et al., 1983, Pan et al., 1986), using all kinds of substrates. *C. curvatus* has great potential because it can use a broad range of substrates including waste products. Most intensively studied was growth of *C. curvatus* on whey permeate. A desaturase mutant of *C. curvatus* which accumulated an oil comparable to cocoa butter was made and production of cocoa butter equivalent (CBE) using whey permeate as a substrate was further optimized on lab scale (Ykema, 1989). In France production of CBE by a *C. curvatus* mutant was studied in continuous culture at various C/N ratios and different dilution rates, using glucose as a substrate (Hassan et al., 1993). The highest lipid production rate (0.42 $gl^{-1}h^{-1}$) was achieved at a dilution rate of 0.123 h^{-1} and a C/N ratio of 24.80 (mol/mol). Results of different fermentations using all kinds of C-sources are summarized in a recent article by Meesters et al. (1995). In this paper we have described growth and lipid production by *C. curvatus* in a fed-batch fermentation with glycerol as carbon source. The fermenter used was a stirred tank reactor (STR) operated at 2000 rpm (maximal agitation) and an aeration rate of 0.6 vvm. The fermentation was divided into two phases. In the first phase all nutrients were in abundance resulting in biomass production while in the second phase nitrogen was limited, leading to the accumulation of lipid. A high cell density of 118 gl^{-1} and a lipid percentage of 25% of total cell dry weight was observed. The overall lipid production rate (LPR) was 0.59 $gl^{-1}h^{-1}$ and during the lipid production phase 1.15 $gl^{-1}h^{-1}$. Growth and product formation during this last phase was linear, indicating oxygen limitation.

Stirred tank fermenters have proven to be not sufficient for fermentation processes that require high oxygen transfer rates. For viscous systems, for example high cell density fermentations, oxygen transfer might become a limiting factor. Therefore a new type of bioreactor was developed by Applikon/Suiker Unie (Oosterhuis and Koerts, 1991). This new reactor called Surer® contains static mixers taking care of oxygen transfer and mixing of the fermentation broth. The mixing time in this fermenter was determined to be 5 times the average circulation time. Higher oxygen transfer rates (OTR) can be obtained by installation of more static mixers. Fermentation in the Surer® reactor was compared to fermentation in conventional stirred tank reactors using the yeast *Candida utilis*. Oxygen transfer rates were best in the Surer® reactor. This was also the case if OTR was measured in water (Olivier and Oosterhuis, 1988).

Lipid production in the Surer® reactor

To asses the applicability of this new reactor type in fed-batch lipid production by *C. curvatus* the fermentation described before was performed in a 3 litre Surer® reactor. To be able to compare both reactor types it was, in first instance, tried to maintain an aeration rate of 0.6 vvm and maximal agitation rates of 35 Hz. However, foam formation in combination with the small headspace of the Surer® reactor forced us to lower the aeration rate to 0.2 vvm and the agitation rate to 20 Hz, resulting in a suboptimal oxygen transfer. Figure 2 shows the production of biomass and lipid in the Surer® reactor. In 72 hours of fermentation a cell density of 91 gl^{-1} was achieved. The fermentation started with a lag phase which lasted the first 20 hours. In this period the biomass concentration increased slowly from 2.0 to 14 gl^{-1}. The reason for this long lag period is not clear, a possible effect of the reactor type can at this moment not be excluded. After the lag period the biomass production phase started in which the cell dry weight increased from 14 to 34 gl^{-1} within 12 hours. During this period a constant pH was maintained by titration with 10% NH_4OH, which also served as nitrogen source. After 30 hours of cultivation 5 N NaOH was used for pH control instead of NH_4OH. This resulted in nitrogen limitation which triggered lipid accumulation. In the lipid accumulation phase the lipid content increased from 9% of cell dry weight at the beginning to 32% of cell dry weight at the end of the fermentation. In this phase 25.6 g lipid is produced in 40 hours of limitation. This corresponds to a LPR of 0.64 $gl^{-1}h^{-1}$ during the second phase of the fed-batch fermentation. The overall LPR was 0.42 $gl^{-1}h^{-1}$, which is lower than the LPR obtained in the stirred tank. The final lipid content obtained in the Surer® reactor was 32% of cell dry weight versus 25% of cell dry weigth in a stirred tank.

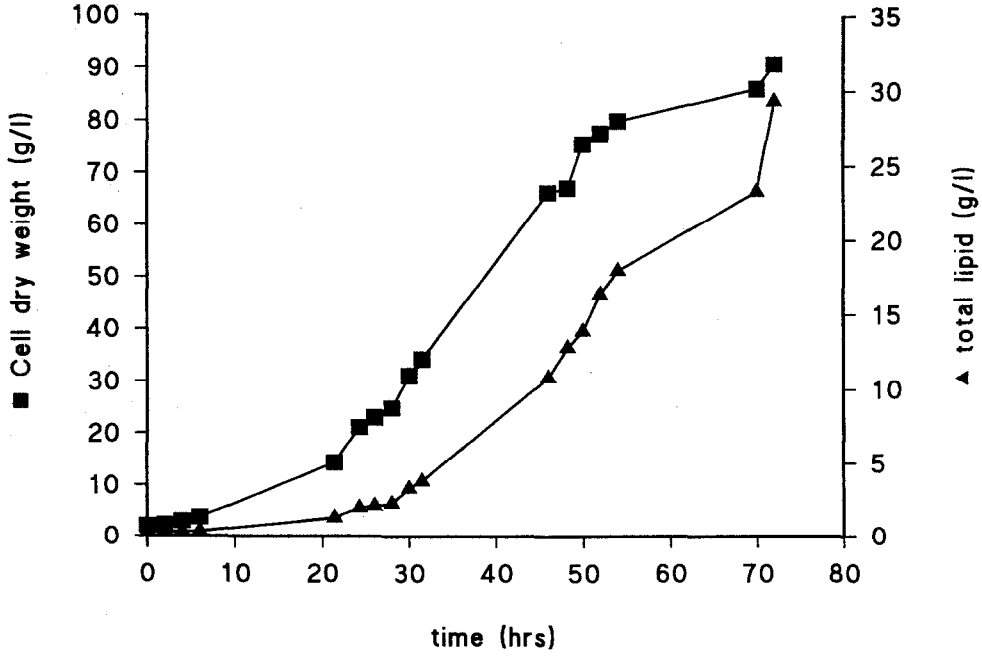

Fig. 2 Cultivation results of Cryptococcus curvatus grown on glycerol in a Surer® reactor.

The results obtained in the Surer® reactor are similar to the values described by Hassan et al. (1993). They reported a fatty acid content of 32% and a lipid production rate of 0.42 $gl^{-1}h^{-1}$ for growth in a continuous culture on glucose. The values found in the Surer® reactor for both LPR and cell density are lower than results obtained in an STR fermentor. This is probable due to a relatively low aeration; 0.2 vvm in the Surer® reactor against 0.6 vvm in the STR. Excessive foam formation made it impossible to use a higher aeration or higher agitation resulting in a sub obtimal oxygen transfer rate in the Surer®.

Fatty acid composition of accumulated lipids in *C. curvatus*

The fatty acid composition of the accumulated lipid was determined and is depicted in Table 1. During fermentation a change in the relative amount of the different fatty acids is observed. During the biomass production phase, in which there is hardly any lipid accumulation, the amount of C18:2(*c*9,12) was high. During the lipid production phase, the amount of C18:2 decreases while the amounts of C18:1(*c*9), C18:0 and C16:0 increased. The composition of the accumulated oil is comparable to the *C. curvatus* lipid composition found in stirred tank fermentation experiments (Meesters et al., 1995).

Table 1. Fatty acid compostion of *C. curvatus* cells during cultivation in Surer® fermenter

	Relative amount of total fatty acids (% wt/wt)				
Cultivation time (hours)	C16:1	C16:0	C18:2	C18:1	C18:0
8	T[a]	15.5	41	38	5.5
24	1	15	40	35	9
48	1	18.5	19.5	47	14
72	1	24	12.5	50.5	12

[a]Trace amounts
C16:1 = palmitoleic acid, C16:0 = palmitic acid, C18:2 = linoleic acid, C18:1 = oleic acid, C18:0 = stearic acid

Scale up of fermentation

In New Zealand, Davies (1992) has worked on the scale up of the fermentation process with *Cryptococcus curvatus*. For industrial purpose an airlift bubble column was used. Comparison of airlift fermenters of 0.5 m^3 and 8.2 m^3 showed LPR's of 0.12 $gl^{-1}h^{-1}$ and 0.06 $gl^{-1}h^{-1}$ respectively. This is much lower than the lipid production rates reported for stirred tank reactors on lab scale. Scale up of fermentation processes in STR's is difficult due to changing mixing characteristics and decreasing oxygen transfer rates. The optimal conditions applied in the set-up described by Meesters et al. 1995 (e.g. 2000 rpm) are difficult to achieve on larger scale, leading to slower production rates and lower final biomass concentrations. The Surer® reactor does not have this disadvantage since its mixing characteristics are independent of the width of the reactor (Fig 1). Oxygen transfer remains constant if liquid velocity, superficial gas velocity, mixer type and the volume of mixers/ total volume, remain constant. Performance of the Surer® reactor was tested at 30 and 4000 litre scale for Xanthan gum production (Oosterhuis and Koerts, 1991). No differences with respect to productivity were observed between 30 l and 4m^3 fermentations. Therefore, we expect that scale up of *A. curvatum* cultivation in a Surer® reactor will result in a lipid production rate comparable to the 0.42 $gl^{-1}h^{-1}$ reported here which is far higher than the 0.06 $gl^{-1}h^{-1}$ reported by Davies (1992).

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