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Optimization of exopolysaccharide production by *Tremella mesenterica* NRRL Y-6158 through implementation of fed-batch fermentation

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In liquid culture conditions, the yeast-like fungus *Tremella mesenterica* occurs in the yeast state and synthesizes an exopolysaccharide (EPS) capsule, which is eventually released into the culture fluid. It is composed of an α-1,3-D-mannan backbone, to which β-1,2 side chains are attached, consisting of D-xylose and D-glucuronic acid. Potato dextrose broth (PDB) seemed to be an excellent medium for both growth of the yeast cells and synthesis of the EPS. This medium is composed solely of an extract of potatoes to which glucose was added. Yet an important disadvantage of this production medium is the presence of starch in the potato extract, since *Tremella* cells are not capable of metabolizing this component; furthermore, it coprecipitates upon isolation of the polymer [3]. In this respect, it was essential to remove the starch in order to achieve high polysaccharide production and recovery. A good method was the removal of starch through ultrafiltration of the PDB medium before inoculation of the strain. This resulted in an excellent starch-free medium in which other components essential for polysaccharide production were still present [3]. Through implementation of single and cyclic fed-batch fermentations with glucose feed, 1.6- and 2.2-fold increases in EPS yield were obtained, respectively. Lowering the carbon source level by using a cyclic fed-batch technique might decrease the osmotic effect of glucose or any catabolite regulation possibly exerted by this sugar on enzymes involved in EPS synthesis.
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Introduction

The yeast-like genus *Tremella* belongs to the family Tremellaceae and to the order Tremellales, parts of the heterobasidiomycetous fungi. Primarily, on the basis of visual observations, the species are thought to be mycoparasites that occur on ascomycetes and basidiomycetes, especially those colonizing recently killed woody twigs, and on lichens [1]. Tremellales and Filobasidiales are the only known taxa with yeast phases. In laboratory culture, isolated basidiospores give rise to yeast states, with mycelia typically developing only when compatible strains are mated.

From several of these yeast-like fungi, polysaccharides have been isolated from either the yeast or the fungus phase. The most important species are *Tremella mesenterica*, *T. fuciformis* and *T. aurantia*. The polysaccharides are characteristic for the various species of *Tremella* that produce them [10] and are of special interest in the medical field. In China, several patents have been filed on ''*Tremella* polysaccharide'' used for cancer prevention and stimulation of the immune system [8,12]. *T. mesenterica* or ''Witches butter'' is a common species in Europe, US (especially the San Francisco Bay Area) and the high mountains of Taiwan. Only few reports have been published concerning the extracellular polysaccharides produced by strain NRRL Y-6158. Fraser *et al* [5] described the synthesis of an acidic polysaccharide by this strain, consisting of D-xylose, D-mannose, D-glucuronic acid and *O*-acetyl in a molar ratio of 7:5:1:0.7, respectively. Slodki [9] reported the existence of an acidic heteropolysaccharide produced by the haploid cells of *T. mesenterica* NRRL Y-6151 composed of mannose, xylose, glucuronic acid and *O*-acetyl in a molar ratio of 4:4:1:0.5, respectively. This polysaccharide resembled markedly the extracellular polysaccharides produced by *Cryptococcus laurentii* NRRL Y-1401 in which the molar ratios of the component sugars were 4:1:1:1.5. The glucuronomannan synthesized by *T. mesenterica* NRRL Y-6151 was also studied by Cherniak *et al* [2], who described it as a (1→3)-α-D-Man*p* backbone, with β-D-Glc*p*A 2 linked to it, as well as 2-linked β-D-Xyl*p* side chains (two or three units long). Further structural studies have not been performed on the exopolysaccharide (EPS) of this strain. A patent for the production of glucuronoxylomannan by *Tremella* spp. was recently granted to Wasser and Reshetnikov [11]. The invention relates to cultivation of a unicellular culture of the edible *Basidiomycetes* mushroom *T. mesenterica* CBS 101939. The glucuronoxylomannans produced by the haploid yeast cells of this species display a hypoglycemic activity. A formulation of dried *Basidiomycetes* biomass was claimed to reduce blood glucose levels. A review on the medicinal value of the genus *Tremella* was recently published by the same authors [6]. *Tremella* glucuronoxylomannan was useful to improve immunodeficiency, including that induced by AIDS, physical stress or aging, and to prevent senile degradation of microvessels [6,7].

We have studied the *T. mesenterica* NRRL Y-6158 fermentation process at shake flask and 2-l bioreactor levels, and aimed at optimization of the production of extracellular polysaccharides

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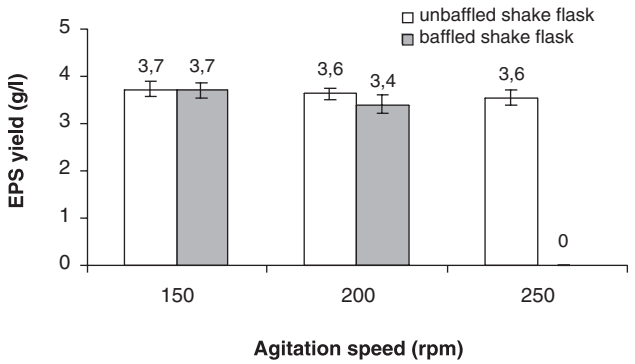

Figure 1 Influence of different agitation speeds on EPS production in filtered PDB in unbaffled and baffled Erlenmeyer flasks (initial glucose level at 20 g/l). In this figure and in Figures 2 and 6, error bars represent the standard deviation between duplicates.

through simple nutritional improvements, i.e., controlled addition of the carbon source *via* fed-batch cultures.

Materials and methods

Culture media and fermentation conditions

T. mesenterica NRRL Y-6158 was grown in potato dextrose broth (PDB; Difco, Detroit, USA) (pH 5.1±0.2), from which starch was removed *via* ultrafiltration. Separation of the starch from the liquid medium was performed using the S1Y100 Amicon Spiral Cartridge, installed in an Amicon Proflux™ M12 ultrafiltration unit [4]. For an efficient separation, the cartridge was rinsed with PDB until the glucose concentration in the medium before and after filtration was equal (no dilution of the medium by water in the cartridge). From that stage, the permeate was collected and used for fermentation optimization. The filtered medium contains 20 g/l glucose.

Fermentations on the shake flask level were performed in 500-ml Erlenmeyer flasks filled with 100 ml of starch-free PDB and closed with hydrophobic cotton plugs. Subsequently, the shake flasks were sterilized by autoclaving them for 21 min at 121°C. Sterilized media were inoculated with liquid 4-day-old corresponding precultures (1% vol/vol), and incubated at 25°C for 7 days on a rotary shaker.

Fermentations in bioreactors were performed in Braun Biostat® M fermentors (2-l reactor vessel), filled with 1.6 l of starch-free PDB. Subsequently, the fermentors were sterilized by autoclaving them for 30 min at 121°C and inoculated with liquid 4-day-old corresponding precultures (5% vol/vol). Fermentations were run at 25°C for 10 days, unless mentioned otherwise. Aeration was set at 1.6 l/min and agitation at 150 rpm without pH control.

Table 1 Effect of the use of baffled shake flasks at high rotation speed (250 rpm) on EPS yield, cell dry weight and residual glucose concentration in starch-free PDB after 7 days of culture

	Unbaffled shake flask, 150 rpm	Baffled shake flask, 250 rpm
EPS (g/l)	3.7	0
Cell dry weight (g/l)	3.5	0.9±0.3
Residual glucose concentration (g/l)*	0	11.98±0.91

*Initial glucose concentration: 20 g/l.

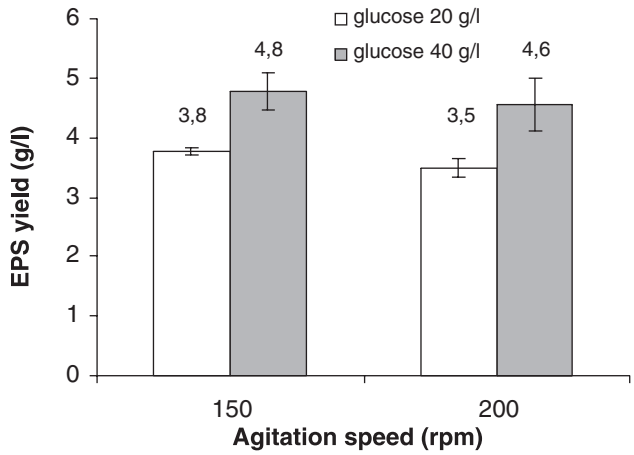

Figure 2 Influence of different agitation speeds and initial glucose levels on EPS production in filtered PDB in unbaffled Erlenmeyer flasks.

Glucose determination

Glucose was determined using the YSI Biochemistry Analyzer 2700 Select, based on an enzymatic oxidation of the glucose present in the sample, followed by electrochemical oxidation of the formed H_2O_2 at a platinum electrode:

$$\text{D-glucose} + O_2 \rightarrow \text{glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-. \quad (2)$$

This produced a probe signal current, correlated with the amount of glucose in the sample. The results obtained are the mean values of triplicate measurements.

EPS recovery

To isolate EPS from the culture broth, cells were removed by centrifugation at 30,000g for 30 min at 4–10°C. Polysaccharides were precipitated from the supernatant by addition of 3 vol of ice-chilled acetone. A subsequent centrifugation at 10,000g for 10 min separated this precipitate from the supernatant, after which it was washed twice with a 60% ethanol solution and dried by freeze-drying.

Cell dry weight (CDW) determination

Aliquots of 5-ml fermentation broth were diluted six times and centrifuged at 30,000g for 30 min at 4–10°C. The supernatant was decanted carefully, and cells were resuspended in 20 ml of distilled water. A second centrifugation was performed at 10,000g for 10 min, after which the supernatant was again carefully decanted. This procedure was repeated. After the second washing of the cells, the pellet was resuspended in a small quantity of distilled water, and transferred to a sand dish (aluminum dish filled with sand), dried and weighed in advance. Subsequently, the sand dish containing the cell suspension was dried at 105°C to constant weight.

Results and discussion

Effect of increased agitation rate and glucose concentration in shake flask cultures

The production of extracellular polysaccharides in the culture broth results in a viscosity build-up, which might limit oxygen transfer to

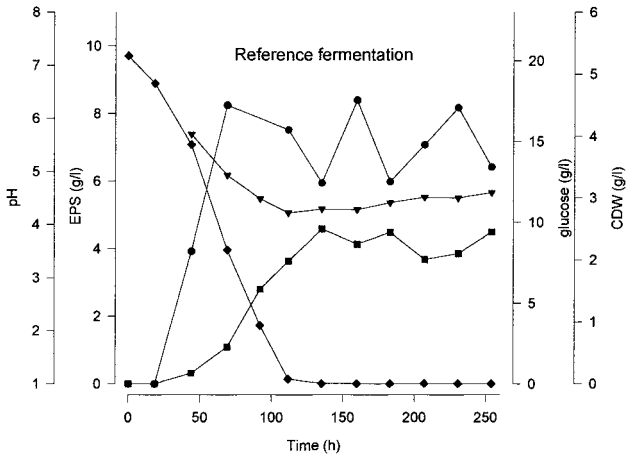

Figure 3 Reference fermentation characteristics: cell growth (●), glucose concentration (◆), EPS yield (■), pH (▼).

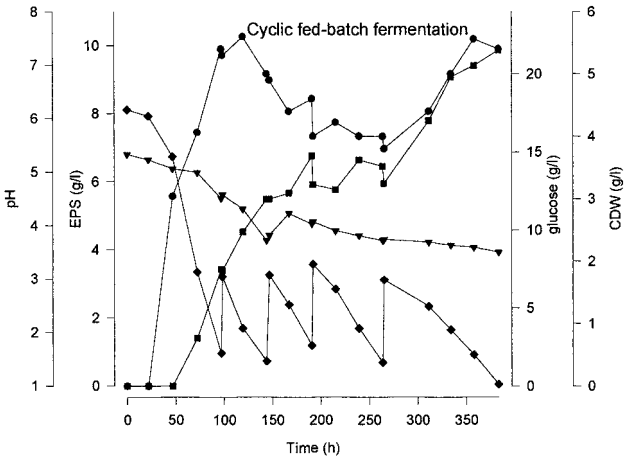

Figure 5 Cyclic fed-batch fermentation characteristics: cell growth (●), glucose concentration (◆), EPS yield (■), pH (▼).

the yeast cells. To verify whether an increased agitation rate — and thus improved oxygen input — affects the polysaccharide yield, three rotation speeds were tested, 150, 200 and 250 rpm, in both unbaffled and baffled shake flasks. Glucose availability rather than agitation speed seemed to be the limiting factor for EPS production (Figure 1). After a fermentation period of 7 days, no significant differences in EPS yield were observed for the different agitation speeds and glucose was depleted from the medium under every test condition. Surprisingly, in baffled shake flasks, no polymer was formed at the highest tested rate (250 rpm). This might be explained by the high shear rates present under these conditions. These results were confirmed by repeating the experiment at 250 rpm in triplicate, showing that cell growth was severely hampered as well (Table 1) at these high shear rates when compared to a reference fermentation in unbaffled shake flasks incubated at 150 rpm.

Since glucose availability seemed to be the limiting factor, the experiment was repeated with a doubled glucose concentration (40 g/l) at rotation speeds of 150 and 200 rpm in unbaffled shake flasks. While EPS production levels at increasing agitation rates up to 200 rpm did not significantly differ at a glucose concentration of 20 g/l, a doubling of the initial glucose concentration increased the EPS yield after 7 days by ±30% (Figure 2). However, in the reference fermentation (initial glucose concentration 20 g/l), glucose was depleted from the culture within 7 days. With the initial glucose concentration raised to 40 g/l, the EPS yield increased by 1 g/l (for both rotation speeds), while only 20% of the extra-added glucose was used (16 g/l residual glucose) within the tested fermentation period. In this context, the specific effect of increased glucose concentration on EPS yield was further examined in bioreactors.

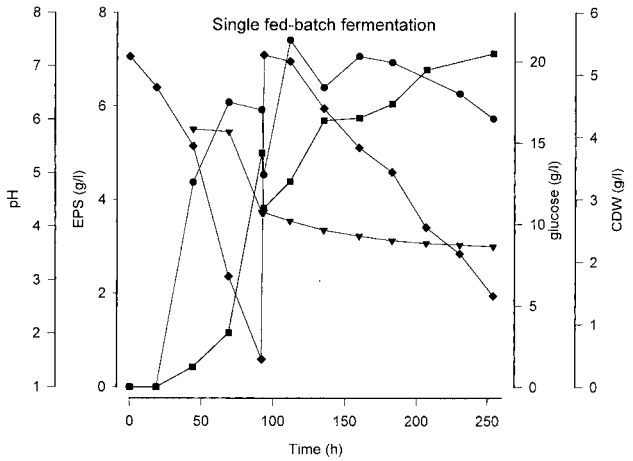

Figure 4 Single fed-batch fermentation characteristics: cell growth (●), glucose concentration (◆), EPS yield (■), pH (▼).

Batch versus fed-batch fermentations

Based on the results of shake flask experiments, the effect of extra glucose addition to the filtered PDB medium was investigated on the 2-l fermentor scale. By implementing the fed-batch culture principle for glucose addition, high osmotic pressures or catabolite regulation effects can be avoided, which may have beneficial effects both on growth and polymer production.

Single fed-batch fermentation: In a first fed-batch fermentor experiment, the glucose concentration was restored to its initial value (20 g/l) upon depletion. This was done by a single-dose addition of glucose (32 g of glucose dissolved in 100 ml of distilled water, added to 1.5 l of remaining culture broth). A reference batch fermentation was run simultaneously. The results of both fermentation profiles are visualized in Figures 3 and 4. The decrease in pH is mainly caused by the acidic nature of the polysaccharide (presence of glucuronic acid residues). In the batch fermentation, glucose consumption was almost completed after 110 h. At this time, an extra glucose feed is added to the culture broth, restoring the glucose concentration to 20 g/l. The consumption rate of the extra added glucose is only half that of the initial glucose (0.1 g of glucose/h versus 0.2 g of glucose/h) and the residual glucose level is 5.6 g/l. For the reference fermentation, polysaccharide production reached a plateau of 4.5 g/l after approximately 135 h. In the fed-batch fermentation, cells continued synthesizing the polymer after the extra addition of glucose and finally, after 254 h, an EPS yield of 7.1 g/l was measured, which means a 1.6-fold increase compared to the reference fermentation.

Cyclic fed-batch fermentation: Implementation of fed-batch fermentation for improving the EPS yield in starch-free PDB indicated that extra addition of glucose to the culture broth has a positive effect. In this respect, this culture technique was further

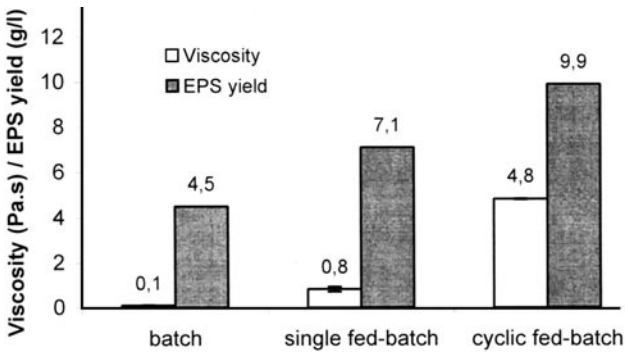

Figure 6 Viscosity of culture broth at the end of fermentation (measured at shear stress; 5 Pa).

examined by carrying out a cyclic fed-batch fermentation. Upon depletion of the initial amount of glucose, the concentration was not restored to its initial value, but to a concentration of 5 g/l. When this amount of glucose in turn was utilized completely, the concentration was again restored to 5 g/l. This procedure was repeated four times, such that in the end, an extra concentration of 20 g/l glucose was added to the culture broth. Immediately after every glucose addition step, small aliquots of the fermentation broth were analyzed since this repeated glucose feed caused an extra dilution of the culture broth. This also explains the irregular shape of the cell dry weight plot. The intervals at which glucose was added to the culture broth are clearly visible in Figure 5. After every feed, the consumption rate of glucose was slightly slower. This cyclic fed-batch fermentation took longer than the two previous ones, since the cells apparently consumed glucose more slowly, requiring 16 days before all the glucose had been used from the medium. The final EPS production in this fermentation run amounted to 9.9 g/l, which means a 2.2-fold increase compared to the reference batch fermentation (4.5 g/l). This yield is even an underestimation due to the dilution effect of the repeating glucose feed.

In conclusion, a cyclic fed-batch fermentation resulted in a higher yield of EPS at the expense of the fermentation time. However, from the same amount of glucose (40 g/l), 2.8 g/l extra polysaccharides are synthesized, just by implementing the repeated fed-batch technique. This may be explained by a decrease in the osmotic pressure of the culture medium, or maybe by preventing a catabolite regulation effect exerted by the rapidly degradable carbon source glucose. However, the latter effect is described to occur mainly in the biosynthesis of secondary metabolites. Very remarkable is the fact that polysaccharide production still continues while cell growth has ceased. This might be an indication that the use of immobilized resting *Tremella* cells can offer an interesting alternative for continuous production of the polysaccharides.

Viscosity measurements of culture fluids

It could easily be observed visually that the viscosity of the fermentation broth in the cyclic fed-batch fermentation was higher than in conventional batch runs. These observations were confirmed by viscosity measurements (Figure 6). This increase in viscosity is correlated with the higher concentration of polysaccharide in the fermentation broth and is again an underestimation due to the dilution of the culture broth. Concentration of the culture broth by evaporation during the long fermentation runs (up to 16 days) was not the cause of this viscosity increase.

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