Effects of Different Aeration Rates and Feeding Strategies on Cell Growth and Invertase Production Kinetics by *Saccharomyces boulardii*

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Invertase (\(\beta\)-D-fructofuranoside fructohydrolase; EC 3.2.1.26) constitutes an important microbial enzyme with wide applications in different food and pharmaceutical sectors. The present work used the biotherapeutic yeast *Saccharomyces boulardii* to produce invertase under different aeration rates in stirred tank bioreactor. Our results showed that an aeration rate of 1 v v\(^{-1}\) m\(^{-1}\) was the most suitable in terms of cell growth and invertase productivity. Highest enzyme production was recorded 14950 U L\(^{-1}\) after 50 h of cultivation. The production process was further optimized using different feeding strategies to overcome substrate limitation side effects encountered during batch cultivation. Sucrose feeding enhanced cell growth and enzyme productivity over batch cultivation by about 56.5% (from 7.35 to 11.5 g L\(^{-1}\)) and 35.5% (from 14950 to 20250 U L\(^{-1}\)), respectively. Furthermore, during feeding phase, invertase production rate was improved by about 62.5% (from 299 to 486 U L\(^{-1}\) h\(^{-1}\)), while growth rate remained constant. Additionally, the improved invertase production was mainly due to increased biomass and not cell productivity, since both batch and fed-batch cultivations have nearly similar specific growth values (2243.9 and 2194.1 U g\(^{-1}\), respectively). On the other hand, feeding complete medium greatly enhanced process parameters. Cell growth and invertase production increased from the batch cultivation by about 143.8 and 120.1% (17.92 g L\(^{-1}\) and 32900 U L\(^{-1}\), respectively), and from the sucrose-feeding cultivation by about 55.8 and 62.5%, respectively.

**Keywords:** Invertase, *Saccharomyces boulardii*, Aeration Rate, Batch, Fed-Batch, Optimization

Introduction

The worldwide growing sector of industrially applied microbial enzymes recorded $ 4.2 billions by 2014, and it is expected to reach $ 6.3 billions by 2020, with a CAGR (Compound Annual Growth Rate) of about 5.8%.\(^1\)\(^2\) Among these enzymes, invertase (\(\beta\)-D-fructofuranoside fructohydrolase, EC 3.2.1.26) is important in different food and pharmaceutical industries.\(^3\) Invertase is mainly used to hydrolyze sucrose and produces the so-called highly sweetening invert sugar, which is non-crystallizable equimolar syrup of glucose and fructose.\(^4\) The annual production capacity of invert sugar worldwide is estimated with 6000 tons (dry weight).\(^5\) Microbial sucrose hydrolysis has been favored over the traditionally used acid hydrolysis due to its higher yield and sweetening power, lower ash and byproduct contents, and stability.\(^6\) Invert sugar is mainly used in many baking, confectionary and beverage industries.\(^9\)\(^1\)\(^2\) Invertase is also used as a plasticizer in cosmetic and paper industries.\(^12\) Additionally, invertases found possible application in the biofuel industry, and are used in the pharmaceutical industry as an ingredient in digestive aid tablets and powdered infnats milk.\(^4\) Moreover, invertase hydrolyzes sucrose outside the cells, thus allowing the produced glucose to cross cell membrane and be ready for metabolic activities inside cells.\(^13\) Recently, invertases possessing fructosyltransferase activities have been used to produce different types of fructo-oligosaccharide (FOS) sugars from sucrose. FOS have many health promoting effects as they work as prebiotics.\(^1\)\(^1\)\(^4\)\(^1\)\(^4\) Invertases have been also reported to decrease blood ammonia and cholesterol levels and prolong patient survival in some cancers.\(^1\)\(^5\) Invertases are generally produced by a wide range of...
microorganisms including bacteria, yeast and fungi. However, the most expensive commercial application has been carried out with yeast strain, i.e. *Saccharomyces* spp. Generally, industrial fermentation processes focus on optimizing the cultivation medium and parameters to achieve highest productivity in terms of cell growth and desired product.

These parameters include medium compositions, cultivation parameters, and cultivation mode. The microbial cultivation process depends mainly on adopting batch and fed-batch modes. Fed-batch is the usual method applied during commercial production of microbiologically valuable products. In our previous work, we optimized batch cultivation processs for invertase production using *S. boulardii* in stirred tank bioreactor. In continuation of our work in the field, the present work was designed to investigate the effect of aeration rate on cell growth and invertase production. Furthermore, the work was extended to compare between batch and fed-batch bioreactor cultivations as well as the effect of different fed-batch modes on cellular growth and invertase production by *S. boulardii*.

**Materials and methods**

**Microorganism**

*Saccharomyces boulardii* ATCC-MYA-796, obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), was used in this study. This strain was adapted to dryness by means of successive adaptation method. Cells were grown on thin film of solid medium and allowed to dry on agar plate at normal cultivation temperature at 30°C. After complete drying of culture, broth medium was added and the cells grew thereafter were isolated for further growth on solid medium. This process was repeated for more than 8 times. The obtained cells were frozen in glycerin solution 50% (v/v) and kept at -80°C until used.

**Growth and production media**

Yeast-peptone-dextrose-agar (YPDA) medium was used to grow cells, and it was composed of (g L⁻¹): glucose, 10; yeast extract, 3; peptone, 3 and agar, 20. The pH was adjusted to 4.5 before autoclaving. The production medium consisted of (g L⁻¹): Sucrose, 30.0; (NH₄)₂SO₄, 5.0; KH₂PO₄, 3.0; MgSO₄·7H₂O, 1.0; yeast extract, 3.0. The initial pH was adjusted to 5.5 before autoclaving. Sucrose was sterilized separately and added to the medium aseptically before inoculation.

**Cultivation conditions**

**Inoculum preparation**

Submerged shake-flask cultivation was used to prepare the inoculum for bioreactor cultivations. Yeast cells were grown in 250 mL Erlenmeyer flasks containing 50 mL of the growth medium. The flasks were incubated on a rotary shaker (Anova 4330, New Brunswick, NJ, USA) at 200 rpm and 30°C for 24 h. Further, the growing cells were used at a concentration of 5% (v/v) to inoculate the bioreactor.

**Bioreactor cultivation**

Bioreactor experiments were carried out in 3.0 L stirred tank bioreactor, Bioflo III (New Brunswick Scientific Co., New Brunswick, NJ, USA), with a working volume of 1.5 L. Agitation was performed using a three 4-bladed Rushton turbine impellers (dimpeller diameter = 65 mm; d tank diameter = 135 mm, d id = 0.48) at 300 rpm. Aeration was performed by injecting filtered sterile air at different aeration rates (0.5, 1.0 and 2.0 v v⁻¹ min⁻¹). Dissolved oxygen concentrations were analyzed by polarographic electrode (Ingold, Switzerland). Foam was suppressed, when necessary, by the addition of silicon antifoam reagent (Fluka, Switzerland). Based on our previous findings, the pH of the bioreactor cultivations was controlled at 5.5 throughout the whole cultivation by the addition of 2.5 Mol L⁻¹ NH₄OH. All the experiments were repeated for three times, and the average mean of the data are represented.

**Analysis**

**Sample preparation and cell dry weight determination**

During bioreactor cultivations, a sample in the form 20 mL of cultivation broth was withdrawn at different time intervals from the bioreactor vessel through the standard sampling nozzle. At the time of sampling, the optical density of the broth was determined at 600 nm after appropriate dilution to measure between 0-0.5 using spectrophotometer (Pharmacia Biotech, Cambridge, England). Samples were then filtered using pre-weighed filter paper and the biomass was washed twice by distilled water and subsequently dried in an oven at 110°C till a constant dry weight is obtained. Cell dry weight was then determined using previously determined standard curve between OD and cell dry weight. The filtrate was then frozen at -20°C and used for further analysis.
Sucrose determination

Residual sucrose was determined using the method of Hagiwara\textsuperscript{25} based on determining total carbohydrates using anthrone reagent. Briefly, cell broth sample (after filtration) was diluted 1:500 by distilled water, from which 1 mL was mixed with 5 mL of 0.2\% anthrone reagent (0.2 g of anthrone in 100 mL conc. H\textsubscript{2}SO\textsubscript{4}). Tubes were put in ice bath for 5 min., and then boiled in water bath for 10 min. Afterwards, tubes were cooled at room temperature for 60 min. and then the developed colour intensity was measured at 620 nm. Sucrose concentration was determined from standard curve.

Invertase activity

The determination of enzyme activity produced in the culture media depended on using a two-step assay method\textsuperscript{26,27}, by measuring glucose liberated from sucrose hydrolysis using an enzymatic colorimetric glucose oxidase-glucose peroxidase kit (Diamond Diagnostics, Egypt). The intensity of developed colour was spectrophotometrically measured at 500 nm. Control tubes were run to avoid the background glucose already present in cultivation broth. One unit of invertase activity is defined as the amount of invertase hydrolyzing sucrose to produce 1 g glucose per 1 min. at 30\(^\circ\)C and pH 4.9. Volumetric activity is used to express units per litre of culture medium [U L\(^{-1}\)], while yield (specific activity, \(Y_{\text{PS}}\)) is defined as units per gram of cell mass [U g\(^{-1}\)].

Results and Discussion

Effects of different aeration rates on the kinetics of cell growth and invertase production by \textit{S. boulardii} in 3.0 L stirred-tank bioreactor

This part of the work was designed to investigate the kinetics of cell growth and invertase production in response to different aeration rates (0.5, 1.0 and 2.0 v v\(^{-1}\) min\(^{-1}\)). From the obtained results presented in Figure 1, it can be observed that under all tested aeration rates cells grew exponentially for the first 20-25 h of cultivation with average growth rate of 0.19, 0.37 and 0.49 g L\(^{-1}\) h\(^{-1}\) at 0.5, 1.0 and 2.0 v v\(^{-1}\) m\(^{-1}\), respectively. Accordingly, increased cell growth rates increased the maximal cell growth obtained at different aeration rates (3.9, 7.35 and 9.8 g L\(^{-1}\), respectively). Additionally, during the exponential growth phase, cells consumed sucrose rapidly with a consumption rate of 1.2, 1.5 and 1.5 g L\(^{-1}\) h\(^{-1}\) at 0.5, 1.0 and 2.0 v v\(^{-1}\) m\(^{-1}\), respectively. Afterwards, sucrose was completely depleted from the cultivation medium, which was reflected on the following decrease in cell growth in all cultivations. Results also showed that both cultivations run at 1.0 and 2.0 v v\(^{-1}\) m\(^{-1}\) were not limited by oxygen dissolved in the cultivation broth, while cultivations run at 0.5 v v\(^{-1}\) m\(^{-1}\) were oxygen limited (data not shown). On the other hand, cells produced invertase with time at production rates of 124.5, 299, 70.3 g L\(^{-1}\) h\(^{-1}\) at 0.5, 1.0 and 2.0 v v\(^{-1}\) m\(^{-1}\), respectively. The highest volumetric invertase production (14950 U L\(^{-1}\)) was obtained at an aeration rate of 1.0 v v\(^{-1}\) m\(^{-1}\) after 50 h, which was about 84.7 and 166.0\% higher than the highest production obtained at 0.5 and 2.0 v v\(^{-1}\) m\(^{-1}\), respectively. Concerning production yield coefficients, it can be noted that the maximal invertase yield coefficients, \(Y_{\text{PS}}\) and \(Y_{\text{PX}}\), (498.3 U g\(^{-1}\) consumed sucrose and 2243.9 U g\(^{-1}\) cells, respectively) were obtained at 1 v v\(^{-1}\) m\(^{-1}\) after 50-55 h of cultivation. For cell growth yield coefficient (\(Y_{\text{YS}}\)), a maximal of 0.33 g cells g\(^{-1}\) consumed sucrose was obtained at 2 v v\(^{-1}\) m\(^{-1}\) after 20 h, mainly due to the increased cell growth obtained at this aeration rate. Results showed that aeration greatly influenced both cell growth and enzyme production. Oxygen is an important nutrient required by aerobically growing cells. Increasing aeration rates is directly proportional to the dissolved oxygen available in the cultivation broth, hence microbial metabolic machinery is more active and cells tend to grow rapidly under highly aerated conditions\textsuperscript{28,29}. However, cellular growth is also dependent on the other available nutrients, and therefore, growth started to decline directly after reaching the end of the exponential phase, mainly due to depletion of nutrients. It has been reported that maximal invertase production occurs during the exponential phase and decreases due to nutrient depletion\textsuperscript{30}. On the other hand, results showed that highest enzyme production was obtained at aeration rate of 1.0 v v\(^{-1}\) m\(^{-1}\). The decreased invertase production at lower aeration rate can be attributed to the absence of sufficient oxygen amounts required for cellular metabolic activities\textsuperscript{24}. Moreover, Pyun \textit{et al.}\textsuperscript{31} reported that yeast cells require sufficient oxygen concentrations for sterols and fatty acid synthesis as well as for efficient protein expression. They also noticed that specific invertase activity decreased rapidly during earlier growth phases when oxygen is not supplied adequately to the culture. However, the highest aeration rate resulted in production of the lowest concentrations of invertase enzyme. This can be mainly attributed to the effect of shear stress on both growing cells and produced enzyme. Silva-
Santisteban and Filho\textsuperscript{32} reported that their inulinase production was greatly affected by shear stress produced from increased agitation and aeration as well as broth rheological properties, which induced cell death and consequently decreased productivity. Additionally, the secreted enzyme has been reported to be also affected by shear stress, and starts to form unfolded denatured inactive protein molecules\textsuperscript{33,34}. This also can explain the obtained decrease in enzyme production at 1 v v\textsuperscript{-1} m\textsuperscript{-1} after reaching maximal invertase production.

\textbf{Fed-batch cultivations of S. boulardii in stirred tank bioreactor with intermittent feeding of sucrose}

The results obtained for batch cultivations clearly showed that sucrose is completely depleted from the
Cultivation broth after 20-25 h. Therefore, the work was further extended to evaluate the effect of using fed-batch mode of cultivation on cell growth and invertase production by *S. boulardii*. The work was designed to investigate the effect of intermittent feeding of sucrose on the production process parameters. Fed-batch cultivations were performed at the optimum aeration rate of 1.0 v v\textsuperscript{-1} m\textsuperscript{-1}. During this experiment, sucrose addition was started at 15 h of cultivation before sucrose depletion in order to avoid cells entering the stationary or decline growth phase. Feeding was based on the average sucrose consumption rate obtained at 1 v v\textsuperscript{-1} m\textsuperscript{-1} during the exponential growth rate (1.5 g L\textsuperscript{-1} h\textsuperscript{-1}). Sterile sucrose solution was intermittently fed every 5 h for the following 35 h, then feeding was stopped and the cultivation mode was switched again into batch mode. The obtained results (Figure 2) showed that for the first 15 h, cells grew exponentially with a growth rate of 0.27 g L\textsuperscript{-1} h\textsuperscript{-1}, sucrose consumption rate of 1.64 g L\textsuperscript{-1} h\textsuperscript{-1} and production rate of 216 U L\textsuperscript{-1} h\textsuperscript{-1}, reaching 4.1 g L\textsuperscript{-1} cells and 3240 U L\textsuperscript{-1}. Once the sucrose feeding was started, cells continued to grow exponentially until the end of the feeding phase, where the growth rate was kept constant, recording a maximal cell growth of 11.5 g L\textsuperscript{-1} at 45 h. Then cell growth started to decrease slightly till the end of the cultivation. On the other hand, during the feeding phase, invertase production rate increased by about 2.25 folds recording 486 U L\textsuperscript{-1} h\textsuperscript{-1}, resulting in a maximal invertase production of 20250 U L\textsuperscript{-1}, which remained more or less constant afterwards. Surprisingly, sucrose started to accumulate in the bioreactor reaching a concentration of 7.4 g L\textsuperscript{-1} at 50 h, and then started to decrease again when the feeding was stopped, and became depleted from the cultivation at 70 h. It is noteworthy to mention that feeding sucrose increased maximal cell growth and maximal volumetric productivity from the batch cultivation by about 56.5 and 35.5%, respectively. Additionally, the invertase yield coefficient (\(Y_{FX}\)) increased with time progress recording 2194.1 U g\textsuperscript{-1} cells, which was comparable to that recorded in the batch cultivation. Feeding is generally performed to overcome cultivation problems encountered due to substrate limitations\textsuperscript{38,39}. Our results showed that sucrose feeding increased both cell growth and invertase production by 56.5 and 35.5%, respectively. These results are in good accordance with those of Valencia *et al.*\textsuperscript{36}, who optimized invertase production by *S. cerevisiae* using fed-batch optimization. However, our results also showed that sucrose started to accumulate in the cultivation medium during the feeding phase, and that invertase production reached its maximal at 50 h, and then remained constant. This can be explained due to feedback inhibition by the accumulating sucrose, or by carbon catabolite repression resulting from increased concentrations of glucose produced in invert sugar by sucrose hydrolysis\textsuperscript{24}. It is well reported that invertase expression in yeast is down-regulated at higher glucose concentrations\textsuperscript{37}.

**Fed-batch cultivation with intermittent feeding of complete medium**

Results of sucrose fed-batch experiments clearly showed that sucrose accumulated during the feeding phase, indicating that sucrose is not the limiting component for production, and that other medium components consumed from the cultivation medium are affecting cellular metabolic activities. Therefore, intermittent feeding of complete medium was performed to improve the cultivation process. This experiment was designed exactly as that with only sucrose feeding, except that complete medium was fed to the bioreactor instead of sucrose. Results obtained (Figure 3) showed that cells behave during the 1\textsuperscript{st} batch phase as normal batch, where cell growth and invertase production parameters were nearly the same as the previously obtained batch results (growth rate, 0.28 g L\textsuperscript{-1} h\textsuperscript{-1}; production rate, 209.3 U L\textsuperscript{-1} h\textsuperscript{-1}; sucrose consumption rate, 1.62 g L\textsuperscript{-1} h\textsuperscript{-1}). Similar to sucrose feeding, the with onset of the feeding process,

![Fig. 2 — Kinetics of cell growth and invertase production during fed-batch cultivations of *S. boulardii* in stirred tank bioreactor using intermittent addition of sucrose](image-url)
cells continued to grow exponentially and reached a maximal cell growth of 17.92 g L\(^{-1}\) by the end of the feeding phase (50 h). Afterwards, cells entered the stationary phase and cell mass remained more or less constant during the 2\(^{nd}\) batch phase. However, it can be seen that the average growth rate during the feeding phase was improved by about 46.4% and recorded 0.41 g L\(^{-1}\) h\(^{-1}\). Contrary to sucrose feeding experiment, the complete medium feeding stimulated growing cells to consume all fed substrates, where sucrose was completely consumed till the end of cultivation and no accumulation was observed. Regarding invertase production, results showed that invertase production rate increased by about 3.6 folds, from 209.3 U L\(^{-1}\) h\(^{-1}\) during the batch phase to 761.7 U L\(^{-1}\) h\(^{-1}\) during the feeding phase. The much higher production rate was reflected on the maximal productivity obtained, which recorded a maximal of 32900 U L\(^{-1}\) at 65 h. It can also be noted, although growth ceased after switching to the 2\(^{nd}\) batch phase, on the other hand invertase production continued for another 15 h, and then reached its plateau after 65 h. During this 2\(^{nd}\) batch phase, production rate dropped again to 206.7 U L\(^{-1}\) h\(^{-1}\), which is almost identical with that obtained in the 1\(^{st}\) batch phase (209.3 U L\(^{-1}\) h\(^{-1}\)).

Results showed that complete medium feeding significantly enhanced growth and production rates, which was reflected on maximal cell growth and invertase production obtained. Feeding with complete medium provides growing cells with essential nutrients required such as nitrogen sources, minerals and trace elements\(^{38,39}\). Furthermore, sucrose metabolism has been found to exhibit a specific physiological response, which is greatly dependent on the presence of suitable nitrogen source in the medium\(^{40,41}\). Accordingly, the presence of adequate amounts of yeast extract is essential in the medium for maximized invertase production, which was obtained upon using complete medium feeding. Our findings can also be confirmed based on the fact that higher glucose amounts in the medium suppress invertase production. Vitolo et al.\(^{37}\) conducted exponential and linear fed-batch cultivations with sucrose solutions. They found that at such conditions, glucose concentration remained at 0.5 g L\(^{-1}\), which was lower than the concentration inhibiting invertase production, and thus, they were able to eliminate the inhibiting effect of glucose. In our results, we can see that during the feeding phase with complete medium, glucose accumulation was prevented and that enzyme production was increased by about 62.5% from the sucrose-feeding experiment.

![Kinetics of cell growth and invertase production during fed-batch cultivations of S. boulardii in stirred tank bioreactor using intermittent addition of complete medium](Image)

**Fig. 3 — Kinetics of cell growth and invertase production during fed-batch cultivations of S. boulardii in stirred tank bioreactor using intermittent addition of complete medium.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Batch (aeration rates, v v(^{-1}) m(^{-1}))</th>
<th>Fed-Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>X(_{max}) [g L(^{-1})]</td>
<td>3.9</td>
<td>11.5</td>
</tr>
<tr>
<td>dX/dt [g L(^{-1}) h(^{-1})]</td>
<td>0.19*</td>
<td>0.247*</td>
</tr>
<tr>
<td>P(_{max}) [U L(^{-1})]</td>
<td>8095.0</td>
<td>20250</td>
</tr>
<tr>
<td>Q(_{p}) [U L(^{-1}) h(^{-1})]</td>
<td>124.54*</td>
<td>486*</td>
</tr>
<tr>
<td>Q(_{s}) [g L(^{-1}) h(^{-1})]</td>
<td>1.2*</td>
<td>1.64*</td>
</tr>
<tr>
<td>Y(_{PS}) [U g(^{-1}) cells]</td>
<td>3627.3</td>
<td>2194.1</td>
</tr>
<tr>
<td>Y(_{PS}) [U g(^{-1}) cons. sucrose]</td>
<td>269.83</td>
<td>1976.3</td>
</tr>
<tr>
<td>Y(_{XS}) [g cells g(^{-1}) cons. sucrose]</td>
<td>0.1462</td>
<td>0.504</td>
</tr>
</tbody>
</table>

* Data taken at the end of the exponential growth phase. n.d.: not determined.

Abbreviations: X\(_{max}\), maximal cell dry weight; dX/dt, cell growth rate; P\(_{max}\), maximal volumetric invertase production; Q\(_{p}\), average invertase production rate; Q\(_{s}\), sucrose consumption rate; Y\(_{PS}\), Y\(_{PS}\), Y\(_{XS}\), yield coefficients.
Conclusion
Table 1 summarized process kinetic parameters obtained for the effect of different aeration rates as well as intermittent feeding strategies using either sucrose only or complete medium (Table 1). Different aeration rates clearly indicate that 1 v v⁻¹ m⁻¹ is the most suitable aeration rate affording higher cell mass and maximal invertase production. Furthermore, applying different feeding strategies can be used to overcome the decreased productivities obtained in batch cultivations. Fed-batch cultivations are superior to batch ones, where they enhanced higher growth and production rates, which were reflected on the improved cellular growth and invertase productivity, by avoiding adverse effects resulting from substrate limitation and unavailability of essential nutrients, cofactors and vitamins.

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References