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Kinetics of Cell Growth and Invertase Production by the Biotherapeutic Yeast, *Saccharomyces boulardii*

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Invertase (β-D-fructofuranoside fructohydrolase; EC 3.2.1.26) is a highly glycosylated enzyme which hydrolyzes sucrose to glucose and fructose. Therefore, it finds many applications in food and feed industries. In addition, this enzyme become more attractive for its potential application in pharmaceutical industries based on its antimicrobial activities and its ability to reduce the side effects of cancer therapy. In this work, the biotherapeutic yeast *Saccharomyces boulardii* has been used as a potential biofactory for extracellular invertase production using semi-defind cultivation medium in different submerged cultivtion systems. The initial results showed that the optimal sucrose cocnentration for invertase production was 30 g L^{-1} which yielded volumetric enzyme production of 6540 U L^{-1}. Further studies for bioprocess optimization were carried out in shake flask, bioreactor cultivations under uncontrolled and controlled pH conditions. The results clearly demonstrated that, scaling up of process from shake flask to bioreactor level increased volumetric enzyme production up to 8111 U L^{-1}. However, this increase was due to the incrase in biomass production (from 3.6 g L^{-1} up to 4.4 g L^{-1}) rather than cell productivity as both cultures showed almost the same specific enzyme production of about 1980 U g^{-1}. Further improvment in the production process was achieved in pH-controlled bioreactor culture (pH 5.5). The maximal invertase production in the controlled culture was increased up to 14830 U L^{-1} concomitant with a significant increase in biomass up to 7.5 g L^{-1}. However, on calculating the specific growth rate, the specific enzyme production was about 2269 U g^{-1}. Thus, we can conclude that the increase in invertase production in bioreactor under controlled pH condition was not only due to the increase in biomass but also due to the increase of cell productivity.

Keywords: *Saccharomyces boulardii*, Invertase Production, Stirred Tank bioreactor, Batch Cultivation

Introduction

Saccharomyces boulardii is a non-pathogenic biotherapeutic and probiotic yeast, which acts as a shuttle liberating effective enzymes, proteins and trophic factors in the interstin to improve immune defenses, digestion and absroption of nutrienst[1,2,3]. Moreover, *S. boulardii* secretes polyamines, mainly spermine and spermidine that regulate gene expression and protein synthesis[4]. *In vitro* studies have demonstrated that *S. boulardii* exerts antagonistic activity against various bacterial pathogens, i.e. *Salmonela enterica* serovars Typhimurium and Enteritis, enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. Coli,* protecting the intestinal tract from diarrheal pathogens and intestinal lesions[5,6,7]. Additionally, *S. boulardii* prevents the Antibiotic Associated Diarrhea (AAD) resulting from long-term antibiotic treatment[8,9]. Whereas wide information was published on the medical and nutritional importance of *S. boulardii,* very little information is available for the industrial production of this type of yeast[10,11]. *Saccharomyces* cells are able to utilize different types of monosaccharides and disaccharides but not able to assimilate starch. This is due to the lack of amlyases enzymes. Cultivation of yeast cells for either Backer's yeast or alcohol production is carried out mainly using either pure sucrose or other sucrose containing cheap raw materials such as molasses[12,13,14]. Yeasts belonging to

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Saccharomyces spp. produce internal and external invertases (β-D-fructofuranosidefructohydrolase, E.C. 3.2.1.26). The external invertase is highly glycosylated and resides in the periplasmic space. By means of invertase enzyme, sucrose is hydrolyzed to glucose and fructose prior being transported across the cell membrane as the first step for sugar assimilation[15]. Invertase has many important industrial applications in the production of confectionery within liquid or soft centers, fermentation of cane molasses into ethanol, in calf feed preparation and also in manufacturing of inverted sugars as food for honeybees[16,17,18,19]. Moreover, invertase has a potential antimicrobial activity[20] and has been recently applied in many cancer therapies to reduce many adverse effects caused by cancer treatments[21].

Invertase is present in many different microorganisms, higher plants and animals, but the most important commercial source is yeast. The industrial production of this enzyme is usually carried out mainly by *Saccharomyces cerevisiae*[22,23]. The aim of the present study was to develop new cultivation strategy for the concomitant production of *Saccharomyces boulardii* cell mass and invertase using sucrose based semi-defined medium in shake flask and bioreactor cultivations.

Materials and methods

Microorganism and growth medium

The yeast strain *Saccharomyces boulardii* ATCC-MYA-796 obtained from (American Type Culture Collection, Manassas, VA, USA) was used in this study. For cell propagation on solid medium, yeast-peptone-dextrose (YPD) medium was used. This medium composed of (g L^{-1}): glucose, 10; yeast extract, 3; peptone, 3 and agar, 20. The pH was adjusted to 4.5 before autoclaving.

Production medium

Semi-defined medium composed of (g L^{-1}): Sucrose, 30.0; $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 3.0.; $MgSO_4.7H_2O$, 1.0 and yeast extract, 3.0 was used for cell mass and invertase production. The pH was adjusted to pH 5.5 before autoclaving. Sucrose was sterilized separately by autoclaving at 110ºC and added to the medium aseptically before inoculation.

Cultivation conditions

Shake flask cultivation and inoculum preparation

In case of shake flask culture, the submerged cultivations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of growth medium. After inoculation, the flasks were incubated aerobically on a rotary shaker (Annova 4330, New Brunswick, NJ, USA) at 200 rpm and 30°C for 24 h. Cells were used thereafter to inoculate either shake flasks or the bioreactor with an inoculum concentration of 5% (v v^{-1}).

Bioreactor cultivation

For bioreactor experiments, cultivations 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 ($d_{i(\text{impeller diameter})}$ = 65 mm; $d_{t(\text{tank diameter})}$ = 135 mm, $d_i d_t^{-1}$ = 0.48)at 300 rpm. Aeration was performed by filtered sterile air [1 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). In controlled pH cultivations, pH was controlled at 5.5 by the addition of 2.5 Mol L^{-1} NH_4OH.

Analysis

Sample preparation and cell dry weight determination

In case of shake-flask and bioreactor cultivations, aliquots of the culture (in form of 5 and 20 mL, respectively) were taken from the flasks or the bioreactor vessel through a sampling system. Cell concentration was determined immediately by spectrophotometer (Pharmacia Biotech, Cambridge, England). The optical density of the culture was determined at 600 nm after diluting the samples into the optical density range between 0-0.5. The samples were then filtered using pre-weighed filter paper and the filtered biomass was washed twice by distilled water and subsequently dried in an oven at 110°C for a constant weight. The relation between OD and cell dry weight was then determined using standard curve between OD and cell dry weight, where one OD unit was equivalent to 0.387 g CDW L^{-1}. The filtrate was then frozen at -20°C and used for glucose determination.

Sucrose determination

Determination of total carbohydrates was carried out by anthrone method according to Hagiwara[24] as follows: one mL of suspension (after cell filtration) was diluted to 500 mL by distilled water, and then 1 mL of diluted medium was mixed with 5 mL of 0.2% anthrone reagent (0.2 g of anthrone in 100 mL conc.

H_2SO_4). The tube was immediately immersed in ice for 5 min., and then heated at boiling bath for 10 min. Afterwards, tubes were held at room temperature for 60 min. and samples were measured at 620 nm. Sucrose concentration was determined from standard curve.

Invertase activity

Invertase activity in the culture media was determined using a two-step assay method[25,26]. The glucose generated from sucrose and the residual glucose in the medium was determined using an enzymatic colorimetric method using glucose oxidase-glucose peroxidase method (Diamond Diagnostics, Egypt). The intensity of developed colour was measured at 500 nm using spectrophotometer (Pharmacia Biotech, Cambridge, England). A parallel blank test was done to eliminate the background glucose in the sucrose preparation. One unit of invertase activity is defined as the amount of invertase which hydrolyzes sucrose to produce 1 g glucose min^{-1} at 30°C and pH 4.9. Volumetric activity is defined as units per litre of culture medium [U L^{-1}] and specific activity as units per gram of cell mass [U g^{-1}].

Results and Discussion

Effect of initial sucrose concentration on cell growth and invertase production by *S. boulardii* in shake flask cultivation

The effect of the initial sucrose concentration (5.0-50.0 g L^{-1}) present in the cultivation medium on the kinetics of cell growth and invertase production was studied in shake flask culture. The inoculated flasks containing the production medium with different sucrose concentrations were incubated for 48 h on rotary shaker at 200 rpm and 30°C. The obtained results Figure 1 clearly showed that initial sucrose concentration greatly affected the production of invertase. Increasing sucrose concentration increased both cell growth and invertase production up to 30.0 g L^{-1}, where the maximal invertase level (5810 U L^{-1}) was obtained. Concomitantly, cell mass was increased with increasing sucrose concentration, and reached 3.4 g L^{-1} at 30.0 g L^{-1}. Afterwards, further increase in initial sucrose concentration showed no positive effect on invertase production, where it decreased gradually and reached 3310 U L^{-1} at the highest tested sucrose concentration (50 g L^{-1}). Furthermore, the invertase yield coefficient ($Y_{P/X}$) increased with increasing sucrose concentration and reached a maximal production yield of 1708.8 U invertase g^{-1} cells at 30.0 g L^{-1}, and then decreased with further increase in sucrose concentration, mainly due to the decrease in the production of invertase. Concerning pH, results showed that the final cultivation pH decreased with increasing sucrose concentration up to 35 g L^{-1}, after which the final pH remained more or less constant around 3.2. The above results coincide well with those previously reported by Ul-Haq *et al*.[23], who reported that the maximal invertase activity produced by different *S. cerevisiae* isolates was obtained upon using sucrose at 30 g L^{-1}. Additionally, they also found that increasing sucrose concentration above 30 g L^{-1} increased both sugar consumption and cell growth while decreased invertase production. This could be attributed to the fact that sucrose concentrations higher than 30 g L^{-1} result in the production of higher levels of invert sugar in the cultivation medium, which consequently produces a glucose-induced repression of invertase production[27]. Furthermore, higher sucrose concentrations produced higher amounts of transformed sugars (glucose and fructose), which have been reported to repress the production of invertase through carbon catabolite repression mechanisms[28,29].

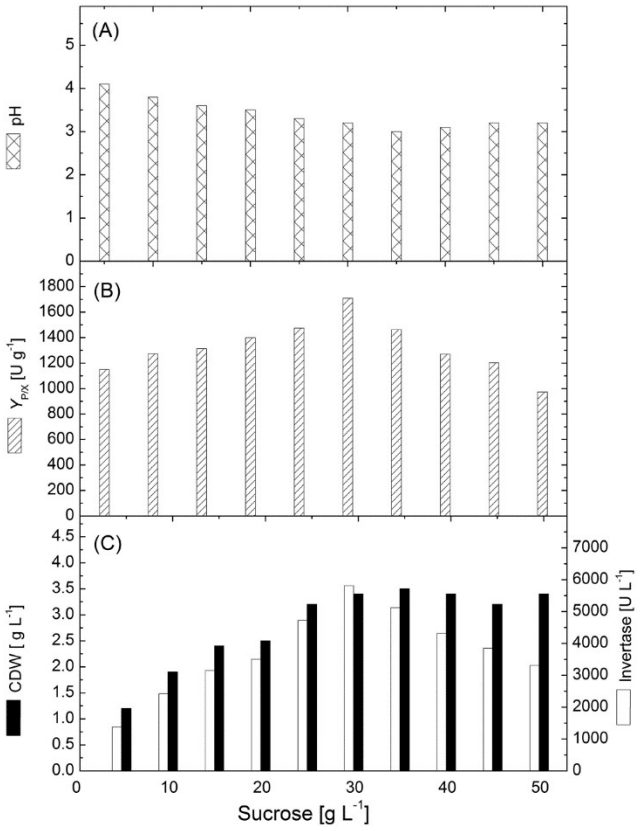

Fig. 1 — Effect of different sucrose concentrations on the cell growth and invertase production by *S. boulardii* in shake flask cultures

Kinetics of cell growth and invertase production by *S. boulardii* in shake flask culture

The kinetics of cell growth and invertase production by *S. boulardii* were evaluated in shake flask cultivation using the optimized cultivation medium containing 30.0 g L^{-1}. Results illustrated in Figure 2 showed that, after inoculation, cells grew during the exponential growth phase for the first 20 h with an average growth rate of 0.18 g^{-1} L^{-1} h^{-1}. During this phase, cells consumed sucrose with an average sucrose consumption rate of 1.5 g^{-1} L^{-1} h^{-1} and they produced invertase with an average production rate of 162.5 U^{-1} L^{-1} h^{-1}. By the end of the exponential phase, cell growth reached a maximal cell mass of 3.6 g L^{-1} and the produced invertase concentration reached 3250 U L^{-1}. During the next 15 h (from 20 to 35 h), cells entered a somewhat stationary phase, where the cell growth remained more or less constant ranging from 3.4 to 3.6 g L^{-1}, mainly due to the exhaustion of sucrose substrate. During this phase the production of invertase continued and reached a maximal concentration of 6540 U L^{-1}at 40 h. Afterwards, invertase production decreased gradually to the end of the cultivation, due to the decrease in cell growth during the cell growth decline phase. During this phase, cells started to die and reached their minimal concentration (2.7 g L^{-1}) at 80 h, where the final invertase concentration decreased to 2640 U L^{-1}. Moreover, at 40 h, the maximal invertase production yields ($Y_{P/X}$ and $Y_{P/S}$) were obtained (1981.8 U invertase g^{-1} cells and 218U invertase g^{-1} consumed sucrose, respectively) due to the production of the highest invertase concentration (6540 U L^{-1}), where they decreased gradually afterwards and reached their minimal yields (977.8 U invertase g^{-1} cells and 88 U invertase g^{-1} consumed sucrose, respectively). Furthermore, during the whole cultivation, the pH decreased gradually and reached a minimum of 2.8 at 25 h and then started to increase up to 4.4 by the end of the cultivation. The obtained results showed that growing cells required a short lag phase of about 5 h to produce invertase, and then reached their maximal production during the stationary growth phase. However, invertase production decreased by the end of this phase. This can be explained on the basis that by the end of the exponential phase, most of the required available nutrients (sucrose, nitrogen source) become depleted from the cultivation medium[23]. Moreover, several other approaches have been proposed to explain this phenomenon; i.e. (1) carbon catabolite repression resulting from increasing glucose and fructose concentrations in the medium, (2) production of growth inhibitors by the yeast cells, and (3) action of protease enzyme during the decline phase[30,31]. Furthermore, the decrease in invertase production could be attributed to the effect of the cultivation pH. Changing the cultivation pH from the optimum was related to the inactivation of glucose-6-phosphate dehydrogenase (G6PDH), which was found to be correlated with biomass accumulation, and hence invertase production[32]. G6PDH is mainly active during the logarithmic growth phase via the pentose pathway, resulting in the synthesis of ribose-6P, which in turn acts as a building block for the synthesis of nucleotides and nucleic acids[33].

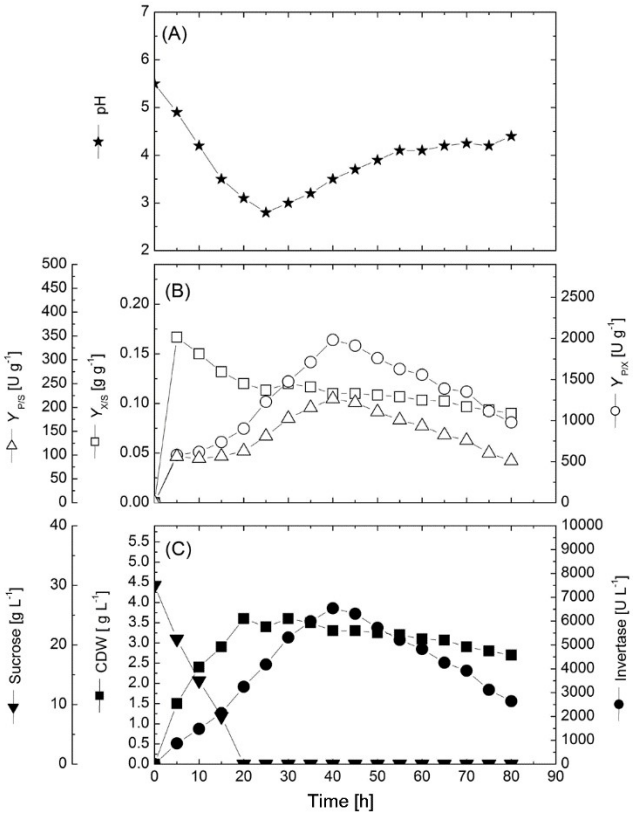

Fig. 2 — Kinetics of cell growth and invertase production during submerged cultivations of *S. boulardii* in shake flask cultures

Kinetics of cell growth and invertase production by *S. boulardii*in 3.0 L stirred tank bioreactor under uncontrolled pH conditions

It is noteworthy to mention that the aforementioned results from shake flask cultivation showed that the pH significantly changed over the course of the cultivation. Therefore, the bioreactor experiments were designed to evaluate the effect of controlling the pH value during the cultivation of *S. boulardii* under bioreactor optimized conditions and its effect on the kinetics of cell growth and invertase production. The

cells were cultivated in 3.0 L stirred tank bioreactor under uncontrolled pH conditions. The obtained results Figure 3, uncontrolled pH clearly showed that the bioreactor cultivation is superior to the shake flask cultivation in terms of improved kinetics of cell growth and invertase production. The cells grew exponentially for the first 20 h of cultivation, where the average growth rate reached 0.22 g^{-1} L^{-1} h^{-1}, which was higher by about 22.2% than the average growth rate obtained during shake flask cultivation (0.18 g^{-1} L^{-1} h^{-1}). The maximal cell growth obtained at 20 h was 4.4 g L^{-1}, which was also higher by about 22.2% than the maximal cell growth obtained in shake flask cultivation (3.6 g L^{-1}). Similar to shake flask profile, cell growth entered a short stationary phase (up to 35 h) where it remained more or less constant, and afterwards decreased gradually to reach 2.8 g L^{-1} by the end of the cultivation. Concomitantly, invertase production increased with incubation time with an average production rate of 263.2 U L^{-1} h^{-1} (higher by about 61.9% than the shake flask rate). The maximal invertase production of 8110 U L^{-1}, obtained at 35 h, was also higher by about 24% than the maximal production in case of shake flask cultivation (6540 U L^{-1}). Furthermore, maximal invertase production yields ($Y_{P/X}$ and $Y_{P/S}$) were obtained at 35 h and recorded 1978.1 U invertase g^{-1} cells and 270.3 U invertase $g^{-1}$of consumed sucrose, respectively. Concerning pH profile, the obtained results showed that the pH decreased during the exponential growth phase from the initial 5.6 to a minimum of 2.8 at 30 h, and then gradually increased to reach 4.6 by the end of the cultivation. During bioreactor cultivation the concentration of the dissolved oxygen (DO) decreased significantly during the exponential growth phase with an average DO consumption rate of 2.28% h^{-1}, and reached a minimum of 43% after 25 h of cultivation. Afterwards, the concentration of DO increased gradually mainly due to the ceasing of cell growth and reached 88% by the end of the cultivation. Scaling up the cultivation process from shake flask level into bioreactor level showed significant improvement in the

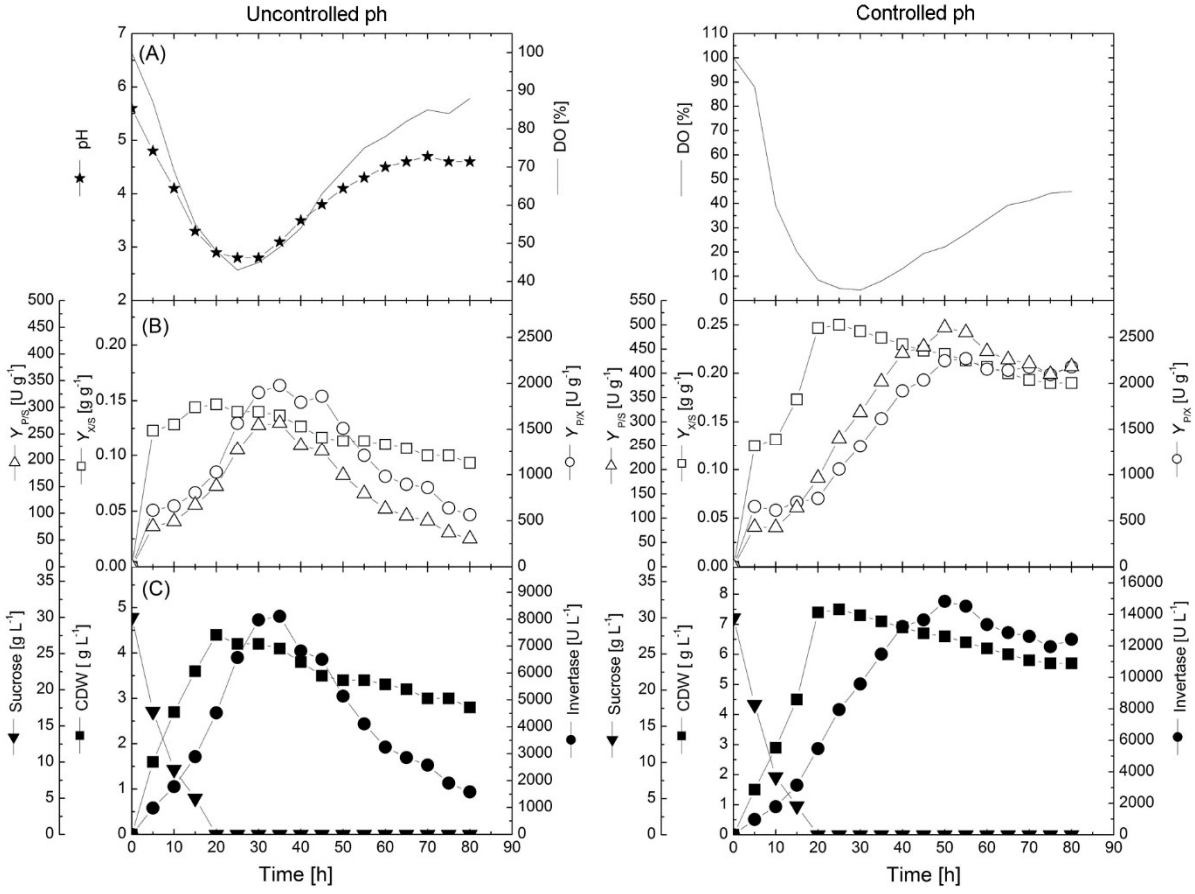

Fig. 3 — Kinetics of cell growth and invertase production during submerged cultivations of *S. boulardii* in 3.0 L STR under uncontrolled and controlled pH conditions

kinetics of cell growth as well as invertase production. This can be generally attributed to the better cultivation conditions present in bioreactor in terms of better mixing and aeration in comparison to shake flask cultivations. This leads finally to improved growth of aerobic microorganisms and the enhanced production of the desired product. This has been proven for different enzymes and microorganisms[34,35]. Singh *et al*.[36] obtained similar results upon transferring the optimized conditions for the production of inulinase by *Kluyveromyce smarxianus*YS-1 from shake flask to bioreactor level. They attributed the higher bioreactor performance to the continuous supply of oxygen and the uniform distribution of medium components in bioreactor cultivation. Furthermore, Shankar *et al*.[28] concluded that the maximal production of invertase takes place in the early growth phases and declines after the exhaustion of medium components and the decrease in the pH of the cultivation. Vitolo *et al*.[27]assumed that the external pH environment correlates with the mechanisms of nutrient assimilation and cellular budding, and hence changing the pH significantly affects cellular growth and invertase production. Moreover, they suggested that the change in the external medium pH can lead to modifications in the tertiary and quaternary structures of invertase, and consequently its deactivation.

Kinetics of cell growth and invertase production by *S. boulardii*in 3-L stirred tank bioreactor under controlled pH conditions

The investigation of the kinetics of cell growth and invertase production under controlled pH condition in bioreactor cultivation revealed that controlling the pH of the cultivation medium at 5.5 over the course of cultivation greatly improved the performance of the bioreactor Figure 3, Controlled pH). The cultivated cells grew exponentially with an average growth rate of 0.37 g L^{-1} h^{-1}, and reached a maximal cell mass of 7.5 g L^{-1} after 25 h of cultivation. Compared to uncontrolled pH cultivation, the average growth rate was higher by about 68.2% and the maximal cell mass was higher by about 70.5%. Furthermore, invertase production was significantly improved, where it was produced with an average production rate of 296.6 U L^{-1} h^{-1} (higher by about 12.7% than the uncontrolled pH cultivation) and reached a maximal of 14830 U L^{-1} after 50 h, which was also higher by about 82.9% than the maximal invertase production under uncontrolled pH conditions (8110 U L^{-1}). Accordingly, the maximal invertase production yields ($Y_{P/X}$ and $Y_{P/S}$) were also improved under the controlled pH conditions and reached 2268.8 U invertase g^{-1} cells and 494.3 U invertase g^{-1} consumed sucrose after 55 and 50 h, respectively. Contrary to the bioreactor cultivation under uncontrolled pH conditions, the concentration of the dissolved oxygen (DO) decreased much more significantly during the exponential growth phase with an average DO consumption rate of 3.19% h^{-1}, and reached a minimum of 8.1% after 35 h of cultivation and then remained more or less constant until 50 h. Afterwards, the concentration of DO increased gradually and reached 45% by the end of the cultivation. The aforementioned results confirmed that controlling the pH during bioreactor cultivation at the pre-set value of 5.5 resulted in a significant improvement in the cultivation process and increased the kinetics of both cell growth and invertase production. Furthermore, growth rate and invertase production rates were greatly improved compared to the cultivation under uncontrolled pH conditions. This could be attributed to the fact that controlling the pH provides the cells with a better physiological environment and promotes the production of invertase[37]. This was also confirmed by the results obtained from dissolved oxygen measurements, where the controlled pH conditions favours active growth and hence, the measured DO concentration dropped to 8.1% after 35 h, whereas it reached a minimum of 43% in the uncontrolled cultivation. On the other hand, although controlling the pH during the cultivation significantly enhanced cell growth and invertase production, however, the cultivation also showed a decrease in the production of invertase after 50 h. This could be attributed to the effect of shear stress on yeast cells as well as enzyme structure. It has been reported that cells tend to die more rapidly under agitated conditions in response to shear stress resulting from impeller blade tips and the broth rheological properties[36]. During bioreactor cultivation, the continuous oxygen supply favouring maximal growth conditions was found to be antagonized by the development of shear stress which affects growth and enzyme yield negatively[38]. Generally, microorganisms differ greatly in their response to the exerted shear stress inside bioreactors during the cultivation as the key factor affecting growth morphology of filamentous microorganisms[39,40,41,42]. However, in case of cultivation of unicellular biofactories, the decreased enzyme activity can be further explained due to the effect of shear stress on the produced protein itself. It

Table 1 — Kinetic parameters of cell growth and invertase production by *Saccharomyces boulardii* under different cultivation conditions

Parameter	Shake flask cultivation	Bioreactor cultivation	
		Uncontrolled pH	Controlled pH
$X_{max.}$[g L^{-1}]	3.6*	4.4*	7.5*
dX/dt [g L^{-1} h^{-1}]	0.18*	0.22*	0.37*
P_{max}[U L^{-1}]	6540.0	8111.0	14830.0
Q_p[U L^{-1} h^{-1}]	162.50	263.20	296.60
Q_{DO}[% h^{-1}]	n.d.	2.28	3.19
$Y_{P/X}$ [U g^{-1} cells]	1981.8	1978.05	2268.75
$Y_{P/S}$ [U g^{-1} consumed sucrose]	218.00	270.00	494.33
$Y_{X/S}$ [g cells g^{-1} consumed sucrose]	0.1667	0.1467	0.2467

* 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_{DO}, DO consumption rate; $Y_{P/X}$, $Y_{P/S}$, $Y_{X/S}$, yield coefficients.

has been well established that protein molecules can't withstand sever changes in cultivation conditions (temperature, pH, solvents, or mechanical stress), and therefore, tend to unfold or denature under these conditions[43]. Although globular proteins are known to be relatively stable in solutions, however, they have been deactivated when subjected to shear stress. Harrington *et al*.[44] reported that the effect of shear stress on the activity of alcohol dehydrogenase, catalase and urease enzymes was negligible when they enzyme solution was affected by shear stress in a coaxial cylinder viscometer designed to eliminate air-solution interface. They found that such viscometer setting prevents air from entering the solution and hence, no vortexing was produced within the enzyme solution. They concluded that the effect of shear stress on protein activity results mainly from the formation of air-solution interfaces, which are generally present in bioreactor cultivations due to continuous oxygenation and agitation.

Conclusion

Table 1 summarizes different kinetic parameters obtained for the shake flask cultivation as well as for bioreactor cultivations under uncontrolled and controlled pH conditions. It can be clearly concluded that the bioreactor cultivation is superior to shake flask cultivation in terms of improved growth and production kinetics. Moreover, controlled pH conditions in bioreactor cultivations promoted enhanced cellular growth and invertase production, by avoiding of the adverse effect of changing pH on growth and protein stability.

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