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Bioprocess optimization for biomass production of probiotics yeast *Saccharomyces boulardii* in semi-industrial scale

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ABSTRACT

Saccharomyces boulardii is non-pathogenic probiotic yeast with many health benefits. In the present work, studies were carried out to improve cell growth kinetics to produce cell mass of this biotherapeutic yeast in semi-industrial scale. At first, five different media were screened to select the most appropriate formula for biomass production. The medium which yielded highest cell mass of 2.57 g L^{-1} and lowest ethanol was selected and further optimized by changing the concentrations of each component. The optimized medium composed of (g L^{-1}): Glucose, 20; Corn steep liquor, 15; $NaNO_3$, 1.0; KH_2PO_4, 6.0; $MgSO_4.7H_20$, 3.0; $CuSO_4.5H_20$, 0.002; $FeSO_4.7H_20$, 0.001; $ZnSO_4.7H_20$, 0.01 was then used for yeast biomass production in a pilot scale 16-L bioreactor in batch culture under controlled and uncontrolled pH. The highest biomass of 8.2 g L^{-1} was achieved in pH controlled culture. This was almost double of the biomass obtained in shake flask. In conclusion, production process has been developed for high cell mass production of the biotherapeutic yeast S. boulardii in semi-industrial scale.

Key words: Probiotic yeast, *Saccharomyces boulardii*, Medium optimization, Semi industrial scale, stirred tank bioreactor

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INTRODUCTION

Probiotics are live microorganisms or microbial mixtures which are applied to improve the patient's microbial balance, particularly the environment of the gastrointestinal tract and the vagina. Therefore, probiotic therapy has been investigated for its effectiveness against a range of gastrointestinal diseases and disorders and became one of the main components of wellness industries [1,2]. *Saccharomyces boulardii*, non-pathogenic yeast that grows optimally at body temperature, has been tested for its efficacy in the prevention of antimicrobial associated diarrhoea. This yeast is commercially available as lyophilised cells in capsule form in many countries. It has recently been used as a dietary supplement in the United States (Biocodex, Inc.). In Germany, the lyophilized form formula is under the trade name Perenterol forte® (Thiemann Arzneimittel, GmbH) and widely used for the prevention of traveller diarrhoea. Beside its probiotic properties, *S. boulardii* is also applied as biotherapeutic agent and used for specific therapeutic activity against many diseases [3]. The yeast *S. boulardii* have showed efficacy in clinical trials for the prevention of antimicrobial associated diarrhoea. However, the potential mechanism of action of this type of yeast is based on its activity for inhibition of pathogen attachment in intestine, inhibition of action of microbial toxin [4], stimulation of immunoglobulin A [5], and trophic effects on intestinal mucosa [6]. Thus, cell mass production of

this type of yeast as high value biotherapeutic/probiotic product was attractive topic for many research groups. Like baker's yeast, *S. boulardii* utilize the carbon source for either cell mass or alcohol production based on the medium formulation and cultivation conditions. Furthermore, a number of studies were carried out for the optimization of *Saccharomyces cerevisiae* biomass production using different media such as completely defined media, semi-defined media and complex media in shake flask and using different processing parameters in bioreactor such as dilution rate, pH, nitrogen limitation, concentration of substrate, and dissolve oxygen rate. Different cultivation processes in bioreactor such as batch, fed-batch were also being investigated to optimize the biomass yeast of *S. cerevisiae* and *S. boulardii* [7-12] and also others bacteria probiotic [13-16]. In the present work, cultivations were conducted in submerged culture to have better understanding on the growth kinetics of cells for high cell mass production in small scale. Moreover, cultivations were conducted to produce the cell mass in pilot scale bioreactors as well. The objective of this research is to study the development of semi-industrial model for high cell mass production of *Saccharomyces boulardii* for biotherapeutic and probiotic applications using different cultivation media and different processing parameters in both shake flask and bioreactor levels. To achieve the objectives, three scopes have been identified in this study, cultivations of *S. boulardii* in shake flask using five different cultivation media, followed by medium optimization study using different concentration of glucose, corn steep liquor and sodium nitrate in shake flask. Finally, studies on the effect of different processing parameters on the production of high cell mass of *S. boulardii* in bioreactor by batch process. Furthermore, the new formulation of this medium had lower cost and thus decrease the overall production cost.

EXPERIMENTAL SECTION

Microorganism

The yeast strain *Saccharomyces boulardii* ATCC-MYA-796 obtained from American Type Culture Collection (Manassas, VA, USA) was used in this study. This strain was adapted to dryness by successive adaptation method [7]. After complete drying of culture, broth medium was added and the cells grew thereafter were taken for further growth on solid medium. This process was repeated 4-5 times.

Preparation of working cell bank

Cells were grown on YPD agar medium, composed of (g L^{-1}): Glucose, 10.0; Yeast extract, 3.0: Peptone, 3.0 and agar 20.0. After 24 hours of incubation at 37 °C, the cells were collected by using 50% (v/v) sterile glycerol. The cell suspension was then transferred to cryovials (1.5 mL each) and stored at -80°C as working cell bank. Every vial was used thereafter as starting stage for inoculum preparation.

Growth and production media

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 5.5 before sterilization. For biomass production, five different types of broth media were used in this study (Table 1). For all media, pH was adjusted to 5.5 before sterilization and carbon sources were sterilized separately and added to the media prior inoculation.

Table 1: Different cultivation media used for screening study

Media	Compositions g L^{-1}	Reference
Medium 1	Glucose,15.0; $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 1.0; yeast extract, 5.0.	[7]
Medium 2	Glucose, 60.0; corn steep liquor, 15.0; $NaNO_3$, 7.0; KH_2PO_4, 6.0; $MgSO_4.7H_2O$, 3.0; $CuSO_4.5H_2O$, 0.002; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.01.	[9]
Medium 3	Sucrose, 40.0; $(NH_4)_2SO_4$, 7.0; KH_2PO_4, 1.0; what bran extract, 200.0.	[11]
Medium 4	Glucose, 80.0; corn syrup, 80.0; urea, 1.0; KH_2PO_4, 6.0; $MgSO_4.7H_2O$, 1.0; $NaNO_3$, 2.0.	[10]
Medium 5	Lactose, 50.0; yeast extract, 2.0; KH_2PO_4, 5.0; $(NH_4)_2SO_4$; 2.0; $MgSO_4.7H_2O$, 0.4.	[12]

Optimization of cultivation medium for higher cell mass production

The best medium supported cell growth was composed of many key nutrients. Of these, glucose, corn steep liquors and sodium nitrates were studied for their effect on cell mass production. Different set of experiments were conducted for cultivating cells in different ratios of these three main nutrients.

Optimization of key nutrients concentrations in Shake Flask

The medium 2 was selected to undergo for optimization process. For optimization experiments, the key nutrients were being identified and prepared at different concentration for three separate experiments. The concentration of

each key nutrient was tabulated in Table 2. Glucose was prepared and autoclaved separately and added to other medium components before inoculation.

Table 2: Optimization of *Saccharomyces boulardii* using three key nutrients (Glucose, Corn Steep Liquor (CSL), Sodium nitrate) in different concentration

Nutrient	Concentrations (g L^{-1})									
Glucose	0.0	10.0	20.0	30.0	40.0	50.0	60.0	70.0	80.0	90.0
CSL	0.0	5.0	10.0	15.0	20.0	25.0	30.0	40.0	50.0	60.0
Sodium nitrate	0.0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0

Cultivation conditions

For shake flask cultures, 250 ml Erlenmeyer flasks with 50 ml working volume were used. After inoculation, flasks were incubated on rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200 rpm and 37°C for 24 hours.

Bioreactor Cultivation

For bioreactor cultures, cultivations were carried out in pilot scale 16-L stirred tank bioreactor bioreactors of 8.0 L working volume (BioEngineering, Wald, Switzerland). Agitation was set at 400 RPM and conducted by two, 4-bladded Rushton turbines. Temperature was controlled at 37°C during the cultivation time and the DO was adjusted to 100% saturation at the beginning of cultivation. For all cultures, the pH was initially adjusted to 5.5 after sterilization and controlled at the same value in case of controlled pH cultures by addition of 2.5M NaOH and 2.5M HCl. The bioreactor was equipped with pH probe, oxygen probe, foam sensor. Sterile antifoam agent (Struktol® J 633, Schill+Seilacher"Struktol" GmbH, Hamburg, Germany) was used during the cultivation process, controlled by low speed feeding pump cascaded with the low-foam sensor.

Analysis

Sample preparation, biomass and ethanol determination

Sample in form of 2 flasks of 50 ml fermentation broth each or 20 ml in case of bioreactor cultivations were taken at different time intervals during cultivation process. For direct biomass determination, small portion of sample was directly diluted using distilled water and optical density was measured at OD_{600} using spectrophotometer (DR/250, Hach Co., Loveland, CO, USA). The yeast biomass was then calculated based on previously prepared standard curve, in which one unit of OD_{600} was equal to 0.33 g L^{-1}. Samples were then centrifuged immediately at 5000 RPM in 50 mL falcon tube. The cell free supernatant was then used for ethanol determination. Analysis of ethanol was carried out using a GC-FID system-Autosystem XL (Perkin Elmer, Norwalk, CT, USA) equipped with a Betadex 120 capillary column (30m, 0.25 mm ID, 0.25µM film thickness, Supelco, Bellefonte, PA, USA). Both the injector and detector temperatures were set at 220 °C and 300 °C, respectively. The GC was equipped with an internal air compressor and hydrogen generator. N2 was used as carrier gas with pressure control (14 PSI). The column temperature program was isothermal at 70 °C, thus giving a total run time of 5 min. Furthermore, 1 µL sample was injected manually. Syringe was thoroughly washed with ethyl acetate between injections to avoid cross-contamination. Chromatography software from Perkin Elmer (Turbochrom 4, ver. 4.1.) was used for data acquisition from the FID.

RESULTS AND DISCUSSION

Effect of different medium composition on cell mass and alcohol production

Based on the previous work of other authors on cultivation of different types of *Saccharomyces* strain, five different media were selected to study their suitability for *S. boulardii* biomass production as described before in materials and methods section. As shown in Figure 1 different media supported cell growth and alcohol production by different extent. Media 1, 2 and 4 included glucose as carbon source while media 3 and 5 have sucrose and lactose as carbon source, respectively. The highest cell mass of about 2.57 g L^{-1} was obtained in medium 2, which composed of glucose, corn steep liquor, $NaNO_3$, KH_2PO_4, $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$, followed by medium 3 which yielded about 1.93 g L^{-1} yeast cell mass. Medium 2 include 60.0 g L^{-1} glucose. The higher amount of glucose can supported high biomass, production of organic acids in small amount and thus lower the pH as consequently. However, it has been also observed that this medium produce alcohol in lower quantities compared to other cultures (except medium 4 which produce very low cell mass and low alcohol as well). Medium which produced high amount of alcohol is unfavorable as this will be on the cost of aerobic yeast growth and biomass production. In addition, ethanol is proved to cause the changes in physiology and in redox balance of

yeast [17]. This resulted in significant reduction of cell growth rate with increasing in ethanol production rate [18,19]. In this screening, medium No. 1 can produce only 1.6 g L^{-1} of cell mass after 24 hours cultivation. The pH for all media was lower than the initial value of pH 5.5 due to alcohol production. Based on these highest biomass productions, medium 2 was selected for biomass production.

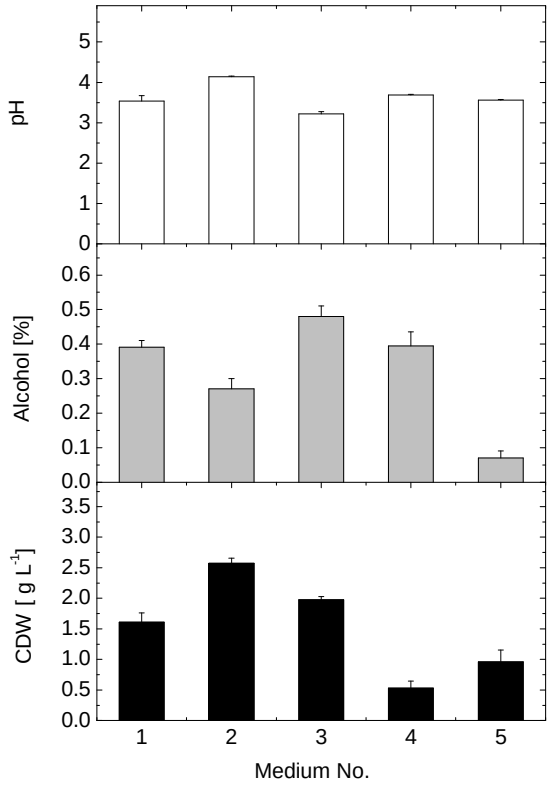

Fig.1: Cell growth and alcohol production by *S. boulardii* on different media after 24 h cultivation at 37°C
The data present mean values and standard deviation of two independent experiments.

Kinetics of cell growth and alcohol production by *S. boulardii* (before optimized)

For better understanding of the cell growth kinetics and alcohol production by *S. boulardii*, cells were cultivated in batch culture in shake flask levels at 37 °C. Cell growth, alcohol production and change in culture pH were followed every hour. Figure 2 showed the typical growth kinetics of *Saccharomyces boulardii* for 24 hours cultivation. According to the study of McFarland and Bernasconi [20], this strain of yeast is able to grow well at high up to 37°C compared to other strains belong to *Saccharomyces sp* who favor lower temperature. As shown in Figure 2, cells grew exponentially with specific growth rate of 0.14 h^{-1} during the first 12 hours cultivation reaching a biomass of 3.25 g L^{-1}. Cells grew thereafter with very low rate until the end of cultivations. This was due to the cells entered stationary phase after 12 hours where the cell growth was limited by the concentration of substrate and accumulation of alcohol. Its also worthy to note that, during the active growth phase (the first 5 hours), the pH of culture dropped gradually reaching 4.0. On the other hand, alcohol was accumulated in culture throughout cultivation time and the maximal alcohol production of about 0.20% was obtained after 24 hours. Thus we can conclude that, the growth was limited after 12 hours cultivations and the rest of cultivation time was mainly for alcohol production.

Effect of different glucose concentration on *S. boulardii* biomass production

In this experiment, the effect of glucose concentration ranging from 0.0 to100.0 g L^{-1} on the growth of *S. blourdii* and alcohol production was investigated. As shown in Figure 3, the biomass increased with the increasing of the initial glucose concentration in culture in the range between 0 and 20 g L^{-1}. The maximal value of obtained biomass was 3.79 g L^{-1} when glucose was added to culture medium in concentration of 20 g L^{-1}. Beyond this concentration, the biomass decreased slowly with the increase of glucose concentration up to 100 g L^{-1}. This showed that after 20 g L^{-1}, higher glucose concentrations did not exhibit any significantly improvement in biomass production of *S.*

blourdii. On the other hand, the alcohol production increased when the concentration of glucose increased even under aerobic cultivation condition. High glucose concentration supported ethanol formation, which indicates metabolic regulation not only by oxygen, but also by glucose through crabtree effect [21]. While the pH for cultivation in different glucose concentration was in a range between pH 4.2 and 4.5. Therefore, we can conclude that the best concentration of glucose for *S. boulardii* growth and biomass production is 20 g L^{-1}. The decrease in biomass production and the increase in ethanol production beyond this concentration was due to the crabtree effect [22].

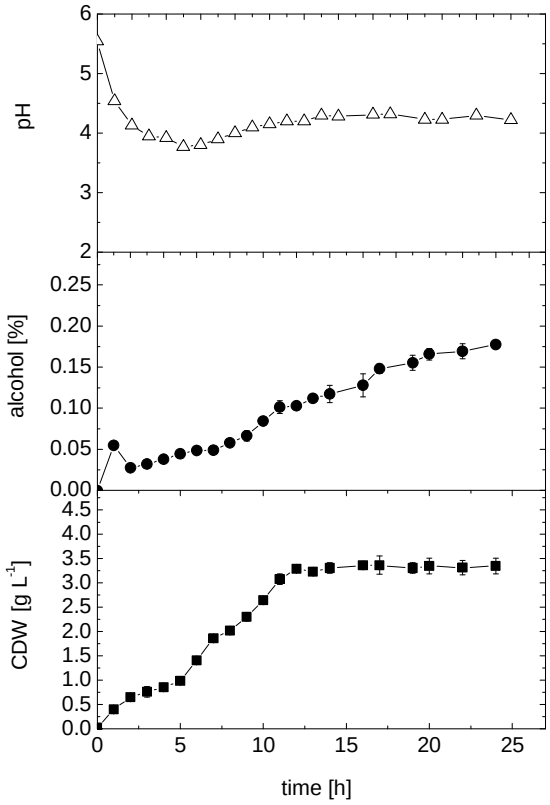

Fig. 2: Cell growth and alcohol production profile of *S. boulardii* in shake flask culture in medium before optimization
The data present mean values and standard deviation of two independent experiments

Effect of different concentrations of corn steep liquor on *S. boulardii* biomass production

Nitrogen sources are very important chemical compound in growth medium has great influence on the yeast biomass. It have been reported that medium supplementation with nitrogen sources such as ammonia, glutamine and asparagin supports higher biomass production during yeast cultivation compared to other sources such as urea and proline [23]. In this study it was clear that addition of corn steep liquor as nitrogen source to medium promoted cell growth of *S. boulardii*. Corn steep liquor is widely used in fermentation medium and not only acts as nitrogen source but also considered as potential source for growth factors, amino acids and salts [9]. In addition, use of low price substrate such as corn steep liquor helps also to reduce the overall production cost espceially in large scale production. In this experiment, different concentrations of corn steep liquor (CSL) ranging from 0.0 to 60.0 g L^{-1} were studied to determine effects of CSL on cell growth and alcohol production. As shown in Figure 4, the maximal biomass production of about 3.6 g L^{-1} concomitant with 0.05 % alcohol accumulation was achieved at 15 g L^{-1} of CSL. However, increasing the CSL concentration from 15.0 to 30.0 g L^{-1} was accompanied by an increased in the production of alcohol from 0.05 up to 0.17% and decreasing cell mass from 3.0 to 2.2 g L^{-1}. Furthermore, it was observed that the addition of CSL in concentration more than 30 g L^{-1} of CSL decreased further the cell mass, of 2.2 to 1.95 g L^{-1}. The maximal value of alcohol production of 0.188 % was obtained in 30.0 g L^{-1} CSL supplemented culture. It is also worthy to note that, the pH was dropped from value of 5.5 to about 4.5 for all concentration of CSL. In conclusion, the best concentration supported of yeast probiotic of *S. boulardii* is 15 g L^{-1} where the addition

of CSL at this concentration highly supported high biomass production and reasonable low alcohol accumulation. Other study by Spigno et al. [24] also reported that corn steep can act as buffering agent in the cultivation medium and can also increase the biomass yield of *S. cerevisiae*. In others study done by Kim and his group CSL was also supportive organic nitrogen source for cell growth of *S. cerevesie* JUL3 and β-glucan production [25].

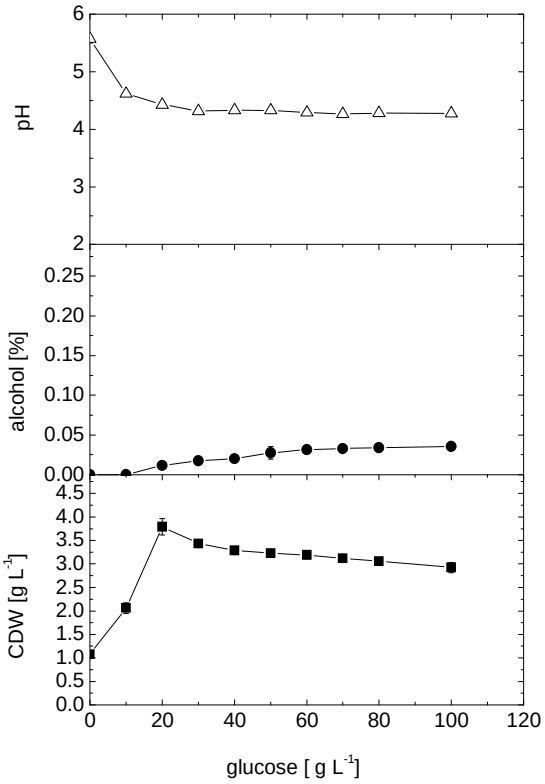

Fig. 3: Effect of different glucose concentrations on the growth of *S. boulardii* in shake flask cultivation

Effect of different sodium nitrate concentrations

The effect of inorganic nitrogen source on cell growth and alcohol production by *S. blourdii* was studied by supplementing different concentration of sodium nitrate to production medium (Figure 5). Sodium nitrate is considered as important inorganic nitrogen source as it was previously reported for its positive role in the biological synthesis of single cell protein and invertase enzyme production during *S. cerevisiae* cultivation process [26,27]. In this study, it was observed that the addition of sodium nitrate in concentration of 1.0 g L^{-1} accompanied by 15.0 g L^{-1} CSL increased the biomass up to 3.51 g L^{-1}. The lowest biomass was about 2.81 g L^{-1} when the cells cultivation in sodium nitrate free medium. However, the addition of sodium nitrate from 2.0 g L^{-1} to 9.0 g L^{-1} did not showed very significant effects on the biomass growth. In general, from the results in figure 5, addition of different concentrations sodium nitrate from 2.0 to 4.0 g L^{-1} contributed to enhance ethanol production rather than cell growth. On the other hand, the highest alcohol percentage reached about 0.15% when sodium nitrate was 4.0 g L^{-1}. However, increasing the sodium nitrate concentration (5.0 to 9.0 g L^{-1}) resulted in significant reduction in alcohol production from 0.08 to 0.02%. For the pH change in culture, all the values were almost the same and in the range of pH 4.2 to pH 4.4. The drop of pH was due to formation of alcohol and other by-products, such as organic acid *by S. boulardii*. From this study it was concluded that the best concentration of sodium nitrate for yeast biomass production is 1.0 g L^{-1}.

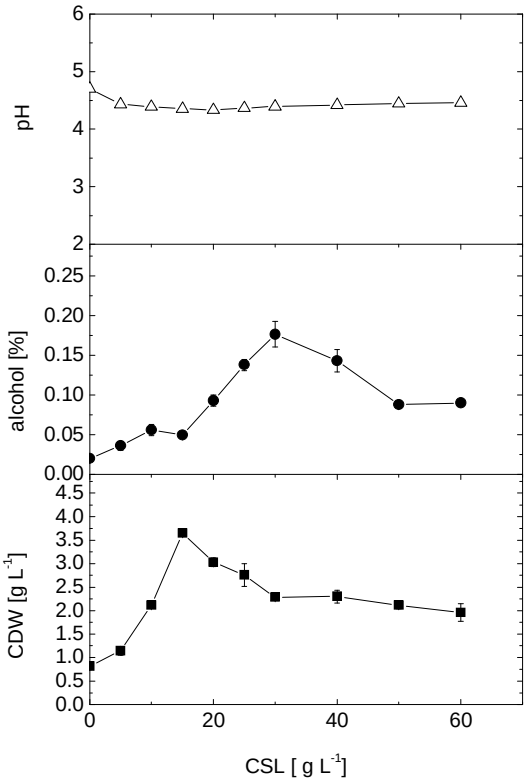

Fig. 4: Effect of different corn steep liquor concentrations on the growth of *S. boulardii* in shake flask cultivation

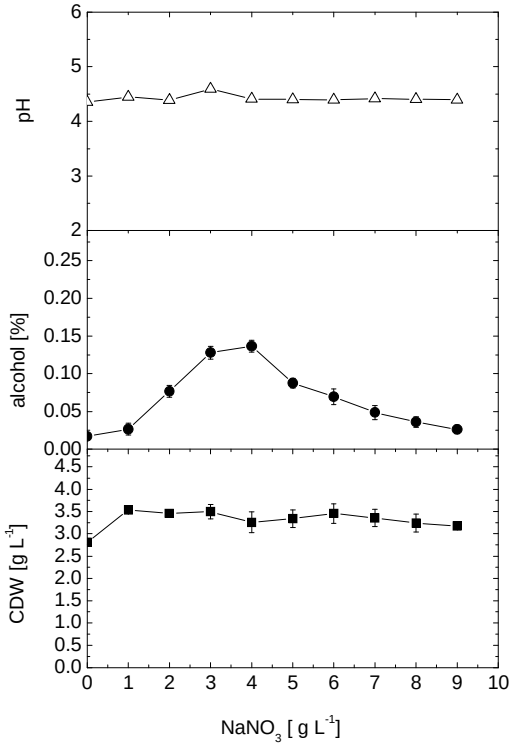

Fig. 5: Effect of different sodium nitrate concentrations on the growth of *S. boulardii* in shake flask cultivation

Kinetics of cell growth and alcohol production by *S. boulardii* using optimized medium

For better understanding of the effect of the optimized medium formulation on the biomass production of *S. boulardii*, batch cultivation was conducted in shake flask level. As shown in Figure 6, cells grew exponentially without significant lag phase and reached biomass 3.9 g L^{-1} after 12 hours cultivation. The biomass kept more or less the same at the same level up to 15 hours of the cultivation and slightly decreased thereafter reaching about 3.5 g L^{-1} at the end of cultivation time. This indicates that the cells reached the stationary phase after 12 hours which may be as a result of accumulation of byproducts and/or nutrient(s) limitation. During exponential cell growth, the medium pH decreased gradually and reached its minimal value of about 4.2 after 12 hours. After that time, the pH remained the same in the range of 4.2 until the end of cultivation. On the other hand, the alcohol accumulated at a very slow rate during the first 12 hours reaching about 0.04%. The alcohol production rate increased gradually thereafter and reached its maximal value of 0.075% after 24 hours. Based on these data, cultivation was conducted in 16-L stirred tank bioreactor using this optimized medium to study the scalability and the industrial potential of this process.

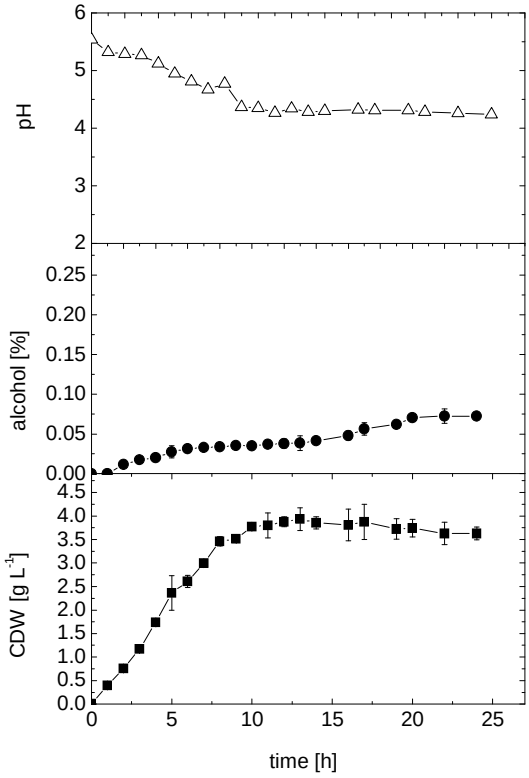

Fig. 6: Kinetics of cell growth, ethanol production and change in pH for optimized medium in shake flask

Kinetics of cell growth and alcohol production in bioreactor 16-L with controlled pH in submerged culture

Batch cultivation was conducted to have better understanding on the kinetics growth, alcohol production and dissolve oxygen consumption during cultivation of cells in semi-industrial scale bioreactor under controlled pH. In this study, cultivation was carried out in 16-L bioreactor with final working volume of 8 L in batch mode under agitation speed of 400 rpm; aeration rate of 1 v/v/min, dissolved oxygen was set at 100% saturation and pH 5.5 at the beginning of the cultivation and maintained throughout the cultivation time by adding 2.5M of NaOH and HCl. As shown in Figure 7, cells of *S. boulardii* grew exponentially with specific growth rate of about 0.14 h^{-1}. The maximal cell mass 0f 8.2 g L^{-1} was achieved after 16 hours and kept more or less constant for the rest of cultivation time in the range 8.0 g L^{-1}. However, the dissolved oxygen decreased rapidly during the first 12 hours and maintain in the range between 35.4% and 37.5% until 24 hours. This indirectly indicates the high oxygen uptake in this culture and active aerobic growth of yeast for biomass production during the first 12 hours. After that time the DO kept more or less constant as cells entered the stationary phase. During active cell growth phase, alcohol production increased gradually in culture until 14 hours. The alcohol production rate was very slow and reached a maximal of

only 0.025% at the end of cultivation time. This low concentration of alcohol production during cultivation time promoted the yeast growth well.

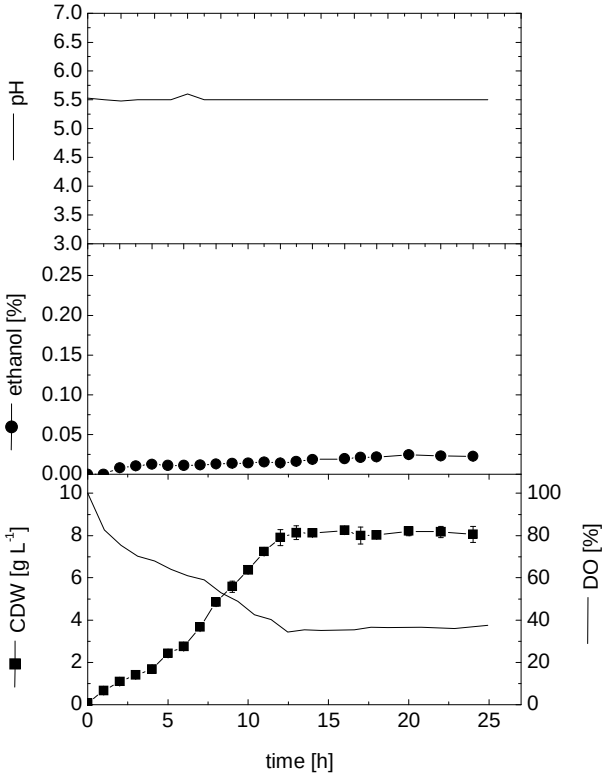

Fig. 7: Kinetic of cell growth, ethanol production and change in dissolve oxygen during batch cultivation of *S. boulardii* in 16-L stirred tank bioreactor under controlled pH

Kinetics of cell growth and alcohol production in bioreactor 16-L with un-controlled pH in submerged culture

In parallel, cultivation using 16-L stirred tank bioreactor was conducted in other bioreactor under un-controlled pH. The pH was adjusted to 5.5 initially. After that, the fermentation was followed every hour for 24 hour. In general, medium was sterilized in bioreactor at 121 °C for 15 min and agitation of 50 rpm, carbon source (glucose) was sterilize separately and added to the bioreactor before inoculation. Cell dry weight, alcohol production and change of pH during cultivations under uncontrolled batch cultivation in 16-L stirred tank bioreactor are presented in Figure 8. As shown, cells of *S. boulardii* grew exponentially with specific growth rate of about 0.18 h^{-1}.The maximal cell growth of *S. boulardii* was about 4.0 g L^{-1} after 13 hours. This value was almost 50% less compared to those obtained in bioreactor culture of controlled pH. This may be attributed to the effect of pH in this culture where lower pH does not support growth and *S. bourlardii* cell mass production. In addition, ethanol concentration in uncontrolled pH culture reached about 0.05% (which almost double of those obtained in controlled pH culture). On the other hand, the pH of the medium decreased dramatically reaching a value of about 4.0 during the first 10 hours. This is might due to ethanol and acid formation. As reported by Muller and his group that the pH of the medium reduced as function of glucose uptake during the exponential phase, which may be attributed to the accumulation of acids in the cultivation medium [28]. The slight pH increased after the active growth phase may due to the formation of ammonia by the free nitrogen from the medium. During the early stage of cultivation, the drop in DO was due to the oxygen consumption of yeast cell during the exponential phase of aerobic growth like the same in bioreactor culture under controlled pH. However, after 12 hours cultivation, the DO increased gradually in culture as the change of oxygen requirements and cells reached the stationary phase. The increase of DO in culture also indicates the less physiological activity during aerobic microbial growth which was a function of the decrease of pH in medium as well [17].

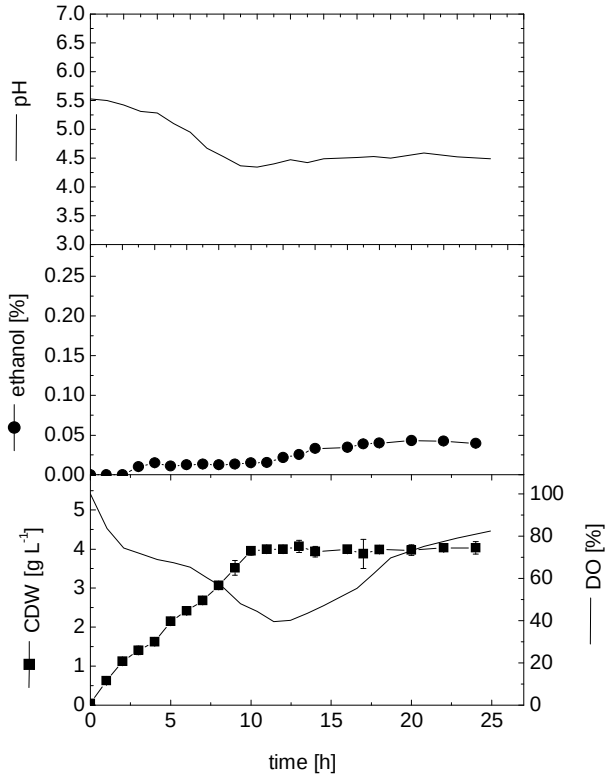

Fig. 8: Kinetic of cell growth, ethanol production and change in dissolve oxygen and pH during batch cultivation of *S. boulardii* in 16-L stirred tank bioreactor under uncontrolled pH condition

Table 3: Medium compositions for *S. boulardii* biomass production before and after medium optimization

Medium components	Initial medium (g L^{-1})	Optimized medium (g L^{-1})
Glucose	60.0	20.0
Corn steep liquor	15.0	15.0
Sodium nitrate	7.0	1.0
Other medium components in both media in (g L^{-1}): KH_2PO_4, 6.0; $MgSO_4.7H_20$, 3.0; $CuSO_4.5H_20$, 0.002; $FeSO_4.7H_20$, 0.001; $ZnSO_4.7H_20$, 0.01.pH 5.5		

Table 4: Summary of growth kinetics parameters in both cultivation in Erlenmeyer flask and bioreactor scale under different cultivation conditions

Parameters	Shake Flask		Bioreactor 16-L	
	Initial medium	Optimized medium	Uncontrolled pH	Controlled pH
X_{max} (g L^{-1})	3.28	3.93	4.00	8.20
Specific growth rate, μ_{max} (h^{-1})	0.14	0.16	0.14	0.18
$Ethanol_{max}$ (%)	0.200	0.075	0.050	0.025
Final pH	4.22	4.24	4.40	5.50

CONCLUSION

In the present work new industrial medium formula was developed for high cell mass production of biotherapeutic/probiotic yeast *S. boulardii*. The composition of medium before and after optimization was presented in Table 3. The optimized medium in this work was composed of (g L^{-1}): glucose, 20; corn steep liquor, 15; $NaNO_3$, 1.0; KH_2PO_4, 6.0; $MgSO_4.7H_2O$, 3.0; $CuSO_4.5H_2O$, 0.002; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.01. Furthermore, cultivations were successfully carried out in pilot scale stirred tank reactor in batch culture to better high cell mass production. As shown in table 4 the highest cell mass of about 8.2 g L^{-1} was achieved after 16 hours when cells cultivated in pH stat culture of 5.5 (this value was almost double of those obtained in uncontrolled pH bioreactor

cultures). However, the advantage of this new medium formula is not only limited to the increase in the yeast cell mass but also to reduce the medium cost.

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