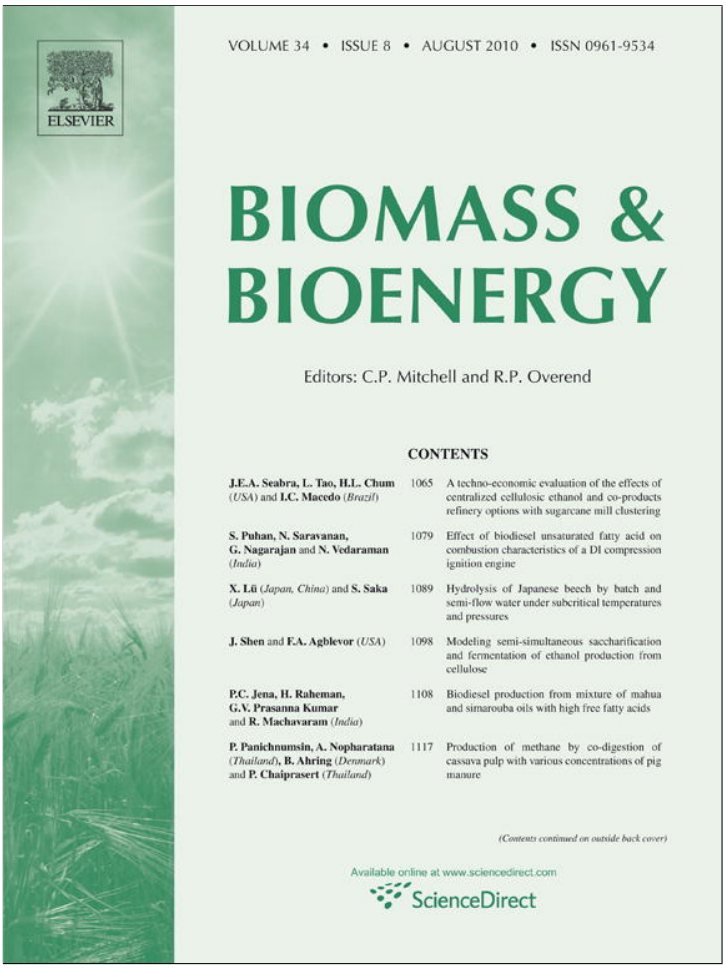

BIOMASS AND BIOENERGY 34 (2010) 1243–1249

Available at www.sciencedirect.com

ScienceDirect

http://www.elsevier.com/locate/biombioe

Production of 16% ethanol from 35% sucrose

*Gaber Z. Breisha**

Department of Agricultural Microbiology, Faculty of Agriculture, Minia University, Minia, Egypt

ARTICLE INFO

Article history:
Received 13 December 2008
Received in revised form
17 March 2010
Accepted 22 March 2010
Available online 15 April 2010

Keywords:
Sucrose
Ethanol
Saccharomyces cerevisiae
Nitrogen
Thiamine
Oxygen

ABSTRACT

A strain of *Saccharomyces cerevisiae*, which showed marked fermentation activity, ethanol and temperature tolerance and good flocculation ability, was selected for ethanol production. A stuck fermentation occurred at sucrose concentration of 25%. Increasing the yeast inoculum volume from 3% to 6% showed positive effects on fermentation from 25% sucrose. The ratio of added nitrogen to sucrose, which gave the best results (for the selected yeast strain), was determined. It was concluded that this ratio (nitrogen as ammonium sulphate at a rate of 5 mg g^{-1} of consumed sucrose) is constant at various sugar concentrations. Addition of nitrogen at this ratio produced 11.55% ethanol with complete consumption of 25% sucrose after 48 h of fermentation. However fermentation of 30% sucrose at the above optimum conditions was not complete. Addition of yeast extract at a level of 6 g l^{-1} together with thiamine at a level of 0.2 g l^{-1} led to complete utilization of 30% sucrose with resultant 14% ethanol production. However the selected yeast strain was not able to ferment 35% sucrose at the same optimum conditions. Addition of air at a rate of 150 dm^3 min^{-1} m^3 of reactor volume during the first 12 h of fermentation led to complete consumption of 35% sucrose and 16% ethanol was produced. This was approximately the theoretical maximum for ethanol production.

1. Introduction

To increase the efficiency of an existing fuel bio-alcohol plant, one potential improvement would be the use of high sugar concentration for fermentation to produce high levels of ethanol. Loss of yeast viability however is a serious problem in fermentation using high sugar concentrations. Limtong et al. [1] reported a decrease in ethanol production at sugar concentrations higher than 22%. They attributed the decrease in ethanol production to various factors including high osmotic pressure and high temperature. Furthermore high ethanol concentrations are produced in the bioreactor systems, the high concentration of ethanol is toxic to yeast growth [2]. In fact, the most expected success in enhancing the yeast's performance and tolerance for ethanol may be achieved by supplementing the media with sources of nitrogen and vitamins. Despite numerous studies are carried out on this topic, the results are not always in agreement and no clear conclusions can be made on the process feasibility. Some authors have reported that nitrogen deficiency reduces biomass concentration and can lead to stuck or sluggish fermentation [3–5]. According to other authors, an excessive nitrogen addition increases the risk for production of acetic acid [5], higher alcohols [6], ethyl carbamate [7], or in some conditions hydrogen sulphide [8]. According to Berthels et al. [9] ammonium addition could counteract turnover of high sugar affinity transporters and also activate phosphofructokinase. As early as 1992, McCaig et al. [10] reported that addition of free amino nitrogen (FAN) leads to higher final ethanol concentrations in the fermented media and higher

* Tel.: +20 2 03 5776848; fax: +20 2 086 2362182.
E-mail address: gaberbresha@yahoo.com
0961-9534/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biombioe.2010.03.017

amounts of accumulated cell mass. They added that yeast growth is proportional to FAN concentrations no higher than 100 mg l^{-1}. For these reasons, the recommended minimal amounts of FAN for an adequate fermentative process are 140 or 150 mg l^{-1} for normal gravity worts, 200 mg l^{-1} for high-gravity wort and 280 mg l^{-1} for very high-gravity wort. Vitamins have protective effects either on yeast growth and fermentation or on viability, which stimulate the fermentation rate and ethanol production [11–13]. Whereas, according to Kaczkowski [14], an excessive concentration of vitamins resulted in deceleration of the fermentation process. Kotarska et al. [15] found that thiamine at a rate of 0.1 g l^{-1} limited the formation of some by-product such as the higher alcohols, and consequently increased the production of ethanol. Also, Dragone et al. [16] concluded that higher ethanol production rates could be achieved by supplementing high-gravity worts with yeast extract (6 g l^{-1}), ergosterol and Tween 80.

Under anaerobic conditions, yeast growth normally requires added oxygen to synthesize lipids (sterols and unsaturated fatty acids), which are essential for plasma membrane integrity. Yeasts under anaerobiosis accumulate unmodified phytosterols (mainly in their esterified form) that can promote yeast growth and initial fermentative activity by acting as substitutes for ergosterol in the yeast membrane. However, in the absence of added oxygen, these sterols quickly perturb the yeast membrane properties by being the predominant sterols, leading to sluggish fermentations [17]. According to Munroe [18] the yeast consumes all of the dissolved oxygen from media usually within the first hours of fermentation. Under this initial aerobic condition, the Embden–Meyerhof–Parnas route (or glycolytic pathway) catabolizes the hexoses to pyruvic acid. The cell completely oxidizes the resultant pyruvate to CO_2 and water while producing energy for other metabolic processes. Only in the absence of oxygen, the pyruvate is converted into ethanol and CO_2 primarily by way of acetaldehyde.

Usually the fermentation efficiency of *Saccharomyces cerevisiae* at high temperatures is very low due to increased fluidity in membranes to which the yeast responds by changing its fatty acids composition. Therefore the use of thermotolerant yeast for alcoholic fermentation is essential [19,20]. On the other hand if the yeast strain is not tolerant to ethanol, some key enzymes in the glycolytic pathway of yeast cells, such as hexokinase and ADH, may be affected by ethanol, and ethanol may also affect the nutrient uptake and cell membrane potential by decreasing the activity of the plasma membrane ATPase [21]. Also Kourkoutas et al. [22] reported that yeast flocculation was a property of major importance for ethanol industry, as it affected fermentation productivity and product quality in addition to yeast removal and recovery. Flocculation can be considered as an immobilization technique as the large size of the aggregates makes their potential use in reactors possible [23]. On the other hand Sharma et al. [24] found that the optimization of inoculum size was necessary as it largely influences fermentation efficiencies. They reported an increase in ethanol production from enzymatically saccharified sunflower stalks, when the inoculum size of *S. cerevisiae* was increased from 3% to 6%. As earlier, Kahlon and Kumar [25] reported only 36 h fermentation time at 8% inoculum size instead of 72 h fermentation time at 3% inoculum size by *S. cerevisiae* for optimum ethanol production from enzymatic hydrolysate of water hyacinth.

It was reported that nitrogen supply must be proportional to the sugar level i.e. increasing the sugar level required a parallel increase in the nitrogen level [26]. According to this criterion, there is a hypothesis that fermentation can be enhanced by nitrogen supplementation. To avoid a decreased synthesis of phosphofructokinase, (a key regulatory enzyme of the fermentation pathway) in excess of nitrogen as reported by Thomas et al. [27], the nitrogen concentration must be optimized. Furthermore Alfenore et al. [28] described a nutritional strategy that allowed *S. cerevisiae* to produce a final ethanol titer of 19% (v v^{-1}) in 45 h in a fed-batch culture at 30 °C. This performance was achieved by implementing exponential feeding of vitamins throughout the process.

The objective of the present study was to produce ethanol by fermentation using a high sucrose concentration. To achieve this objective an efficient strain of *S. cerevisiae* was selected and the inoculum, nutrients (nitrogen, vitamins and yeast extract) and oxygen levels were optimized.

Table 1 – Selection of the most efficient yeast strain according to fermentation activity, ethanol and temperature tolerance and flocculation ability.

Strain No.	Source	Produced ethanol (%)	Cell no at 15% ethanol ($\times 10^6$ ml^{-1})	Cell no at 35 °C ($\times 10^6$ ml^{-1})	Flocculation ability
1	Ain-Shams Univ.	3	21	71	+
2	Cairo Univ.	4	13	67	+++
3	Minia Univ.	4	45	80	++
4	Pressed baker's yeast – Alexandria	3	22	23	++
5	Pressed baker's yeast – Cairo	5	67	56	+++
6	Pressed baker's yeast – Minia	5	70	97	++++
7	Pressed baker's yeast – Sohaj	8	100	137	++++
8	Commercial dry yeast	2	0.0	35	++
9	Commercial dry yeast	3	20	83	++
10	Commercial dry yeast	2	0.0	38	+++
11	Isolated from beer	5	35	91	+++
12	Isolated from beer	4	47	77	++

Each value is the mean of three replicates.

2. Materials and methods

2.1. Microorganism

Ten strains of *S. cerevisiae* were obtained from different locations as indicated in Table 1. In addition, two yeast strains were isolated from the flocculated biomass in two different bottles of beer. Isolation was carried out at 35 °C by an enrichment technique using sugar juice media containing sugar cane juice (5% or 8% total sugars), 0.05% $(NH_4)_2SO_4$ and 4% (v v^{-1}) ethanol with pH of 4.5. After inoculation, cultures were incubated for 3 days in a rotary shaker (Gallenkamp Orbital Incubator, Leicester, UK) at a predetermined temperature with shaking speed of 2.5 Hz. Enriched cultures were then streaked on agar plates containing the same medium and incubated at 30 °C. All yeast strains were microscopically examined, kept on YM slants and stored at 4 °C.

2.2. Fermentation tests

The twelve yeast strains were reactivated in sterile YM broth, incubated in a shaker at 30 °C and 1.67 Hz, for 24 h. Selection of the most fermentative yeast strain was performed in a molasses medium with 18% fermentable sugars, non-sterilized, without nutrients supplementation or pH adjustment, in Erlenmeyer flasks with screw caps incubated in a shaker at 1.7 Hz and 30 °C, for 72 h. The strain that would produce higher amounts of ethanol was chosen for the study. Yeast inoculum was previously cultivated in diluted molasses (8% fermentable sugars, not sterilized) supplemented with sources of magnesium ($MgSO_4 \cdot 7H_2O$) and nitrogen (NH_4NO_3), at 30 °C and 1.7 Hz, for 12 h. Biomass concentration at the beginning of fermentation was adjusted to 1×10^8 viable cells ml^{-1}. This was achieved by inoculation of molasses media with the prepared inoculum at a rate of 4%. Ethanol concentration, yeast cell number and residual sugars were determined every 12 h.

2.3. Ethanol tolerance test

The reactivated yeast strains were submitted to tests of ethanol tolerance. Colonies were transferred to YM broth medium supplemented with different concentrations of ethanol (10%, v v^{-1}; 15%, v v^{-1}; or 20%, v v^{-1}) and incubated at 30 °C for 3 or 4 days. The viable cell populations of each strain were enumerated by pour plate counts on YM agar medium. Successful cultures were collected and screened further for their temperature tolerance.

2.4. Temperature tolerance test

Ethanol-tolerant cells (up to 15% v v^{-1} ethanol) were transferred to plates containing YM medium plus ethanol (15%, v v^{-1}) and incubated at different temperatures (30 °C; 35 °C; and 40 °C) also for 3 or 4 days. The viable cell populations of each strain were enumerated by pour plate counts on YM agar medium.

2.5. Measurement of flocculation ability

Flocculation was estimated by eye. This consisted of viewing the sides and bottom of the culture flasks against the ceiling light. Then expressed as (−) when no gross floc was observed, (+) for a culture which showed any flocs at all, and (+) followed by some number of + (from 1 to 3) for highest degree of flocculation.

The most fermentative, flocculated, ethanol- and temperature (up to 35 °C)-tolerant strain was selected to continue the process.

2.6. Maintenance and inoculum preparation

The selected *S. cerevisiae* was maintained on YM agar slants containing (g l^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 20. The medium used for inoculum preparation contained (g l^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10. Glucose was autoclaved separately at 120 °C, 15 min. Inocula were prepared by transferring one loop full of 24 h culture grown on a slant of YM agar to a 1000 ml Erlenmeyer flasks containing 200 ml of the inoculum medium. Cells were cultivated at 2.3 Hz; 30 °C for 24 h.

2.7. Analysis

Appropriate dilutions from the growing culture suspensions were made and the pour plate counts on YM agar medium was used for counting the viable yeast cells. For ethanol analysis cell-free samples were obtained by sterile filtering of the growth medium using 0·22 μm filters (Cameo 25AS, Micron Separations Inc., Westboro, USA). Samples were subsequently stored at −20 °C until analysis. Ethanol production was determined by using a potassium dichromate method, as described by Salik and Povoh [29]. This determination was quite sensitive and consisted of oxidizing ethanol of the fermented media to acetic acid in a known quantity of acidic potassium dichromate as an acceptor. The excess of potassium dichromate was titrated with ammonium iron (II) sulphate in the presence of the diphenylamine indicator. The maximum theoretical ethanol yield from sugar was calculated according to the stoichiometric relation represented by the equation $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$. This relationship shows that 100 g of a hexose produce 51.1 g of ethanol and 48.9 g of CO_2. Total reducing sugars (as glucose) were determined by the phenol sulphuric acid method using a Shimadzu UV-120-02 Spectrophotometer at 488 nm as described by Herbert et al. [30].

2.8. Fermentations

The conventional batch fermentation processes were performed by using a 5 liter fermenter (INFORS AG Rittergasse 27 CH-4103 Bottmingen, Switzerland) with 3 liters working volume. Artificial basic media, which consisted of (g l^{-1}): yeast extract 3, $(NH_4)SO_4$ 0.5, $K(PHO_4)_2$ 1.5, $MgSO_4 \cdot 7H_2O$ 1.5, peptone 5 and the desired concentration sucrose were used. Levels of yeast extract and ammonium sulphate were changed during the study of their effects on fermentation activity. Thiamine was also added at the desired concentration during studying its effect as fermentation activator. The glass vessel of the fermenter containing media were autoclaved for 15 min at 120 °C after, then the desired level of yeast inoculum suspension was added. The fermentation was allowed to

Table 2 – Effect of increasing sucrose concentration on ethanol production, viable yeast cell numbers, fermentation time and consumption of sucrose by *Saccharomyces cerevisiae*.

Sugar concentration ($g\,l^{-1}$)	Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1})	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
50	24	150	0.0	2.40
100	24	155	0.0	5.00
150	38	120	0.0	6.80
200	72	100	0.0	8.70
250	72	22	130	4.50

Each value is the mean of three replicates.

proceed at 35 °C at an agitation speed of 2 Hz. The pH was automatically adjusted to 4.5 by 2 mol l^{-1} NaOH. Samples were withdrawn aseptically from the fermenter periodically for analysis of total sugar, ethanol and viable yeast cells.

3. Results and discussion

3.1. Selection of the most efficient yeast strain

The results in Table 1 suggested that strain number 7 could be used for ethanol production from high sugar concentrations. This strain showed marked fermentation activity, high ethanol and temperature tolerance and very good flocculation ability, as compared with the other strains. These characteristics are very important for successful fermentation. Based on the results in Table 1, strain number 7 was selected for subsequent experiments.

3.2. Fermentations

The fermenter contained 3 l of the fermentation medium which was inoculated with 3% of high density inoculum cells (about 3×10^8 cm^{-3}). This experimental process was used with increasing the sucrose concentration until stuck fermentation occurred. The time course of ethanol fermentation, residual sucrose, yeast cell numbers and ethanol concentration are illustrated in Table 2. These results showed an increase in the final ethanol concentration with complete assimilation of sucrose up to 20%. The results showed a stuck or sluggish fermentation at sucrose concentration of 25%. The viable yeast cells decreased during the fermentation suggesting that the yeast cells suffered from inhibition and/or osmotic stress. It is thus likely that the sugar consumption for cell growth was very low and only 4.5% of alcohol was produced with a 12–13% residual sugar concentration. It can be concluded that the value corresponding to the substrate inhibition for the yeast strain under test was 25% sucrose concentration.

Table 3 – Effect of increasing the inoculum volume to 6% on alcoholic fermentation of the basic medium contains 25% sucrose concentration by *Saccharomyces cerevisiae*.

Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1}	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
0.0	2	250	0.0
12	45	120	3.0
24	76	98	5.5
36	98	87	7.0
48	100	68	9.3
60	100	60	9.0
72	66	60	9.2

Each value is the mean of three replicates.

3.3. Effect of increasing the inoculum volume

The results in Table 3 showed a positive effect on fermentation of 25% sugar concentration and higher viable yeast numbers along the fermentation period as a result of increasing the inoculum size from 3% to 6%. Due to the fast cell growth within the reactor most of the substrate was immediately converted to ethanol, which reached 9.3% representing about 73% of the theoretical value. This may be attributed to the short or negligible lag phase. The accumulation of viable cells in the bioreactor reduced the fermentation time from 72 h previously found with the 3% inoculum volume to 48 h with the 6% inoculum volume.

3.4. Effect of nitrogen supplementation

We calculated the ratio of added nitrogen to consumed sucrose ($mg\,g^{-1}$), which gave the best results in this work. This ratio was 5 mg nitrogen as ammonium sulphate g^{-1} of sugar. For fermentation of high sugar concentrations, we suggest that this ratio should be nearly constant. Increasing the added ammonium sulphate level to 1250 $mg\,l^{-1}$ of medium made this ratio right for fermentation of 25% sucrose (250 $g\,l^{-1}$). The results of fermenting 25% sucrose after supplementing the production medium with this quantity of ammonium sulphate are illustrated in Table 4. Complete consumption of sugar was achieved and 11.5% ethanol representing about 90% of its theoretical value was produced. The multiplication of yeast cells was greatly activated and reached its maximum value (134×10^6 cfu ml^{-1}). In this work we calculated amount of the nitrogen as ammonium sulphate which, is required to ferment a certain sugar concentration. To confirm this calculation it was decided to try this level of nitrogen at higher sugar concentration (30%). The results (data not shown) confirmed our calculation and 10.2% of ethanol being about 67% of the theoretical maximum value. However a considerable amount of sugar (56 $g\,l^{-1}$) was left unfermented. This may be due to a deficient in other nutrient factors such as vitamins.

Table 4 – Effect of adjustment of nitrogen: sugar ratio (5 mg ammonium sulphate g^{-1} of sugar) on fermentation of 25% sucrose concentration by *Saccharomyces cerevisiae*.

Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1}	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
0.0	2	250	0.0
12	21	180	2.0
24	63	94	4.4
36	97	63	8.7
48	134	0.0	11.5
60	120	0.0	10.5
72	53	0.0	10.00

Each value is the mean of three replicates.

3.5. *Effect of vitamins*

The results in Table 5 showed that thiamine at a rate of 0.2 $g\,l^{-1}$ greatly enhanced the fermentation efficiency of 30% sucrose and ethanol concentration reached 12% being about 74% of its theoretical value. Thiamine also increased yeast viability and sugar consumption. As not expected the addition of thiamine at higher concentration (0.3 $g\,l^{-1}$) gave a negative effect on ethanol production (data not shown) although its effect on yeast growth or sugar consumption was positive. This is similar to the finding of Kaczkowski [14] who reported that thiamine caused improvement in fermentation through the enhancement of the growth of yeast cells, whereas, its concentration above 200 $mg\,l^{-1}$ caused deceleration of the process.

As a source of vitamins yeast extract was added at 6 $g\,l^{-1}$ together with thiamine (0.2 $g\,l^{-1}$). The results of this experiment are illustrated in Table 6. These treatments directed the fermentation pathway to production of ethanol with complete utilization of the sugar and great increase in the viable yeast cell numbers. Under these conditions the ethanol concentration from 30% sucrose was 14% representing about 91% of its theoretical value. These results are comparable to the results of Alfenore et al. [28].

Under the above optimized conditions attempt was made to ferment a medium with 35% sucrose concentration. However the selected yeast was not able to grow well and only 5% of ethanol was produced with high amount (20%) of residual sugar.

Table 5 – Effect of adding thiamine (0.2 $g\,l^{-1}$) on fermentation of 30% sucrose medium by *Saccharomyces cerevisiae*.

Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1}	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
0.0	2	300	0.0
12	26	200	2.4
24	52	139	7.2
36	87	100	9.7
48	102	44	12
60	97	74	11.8
72	80	62	10.4

Each value is the mean of three replicates.

Table 6 – Effect of adding yeast extract (6 $g\,l^{-1}$) and thiamine (0.2 $g\,l^{-1}$) on fermentation of 30% sucrose medium by *Saccharomyces cerevisiae*.

Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1}	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
0.0	2	300	0.0
12	20	210	2.6
24	86	121	8.5
36	98	71	10.6
48	135	0.0	14
60	112	0.0	11.8
72	34	0.0	11.5

Each value is the mean of three replicates.

3.6. *Effect of oxygen*

Under the fermentation conditions a small amount of oxygen is required for yeast cells to synthesize ergosterol and the unsaturated fatty acids which are essential for plasma membrane integrity [31,32]. Accordingly a small amount of air (150 $dm^3\,min^{-1}\,m^3$ of reactor volume) was added during the whole period of fermentation of 35% sucrose medium. The results showed that sugar concentration decreased by 100%, while the cell population increased from 2 millions to 172 millions ml^{-1} after 48 h of fermentation and nearly no ethanol was produced (Table 7). Since oxygen should be added only during the period in which pyruvic acid is formed [18] we added the above amount of air only during the first 12 h of fermentation. The results of this trail showed a complete consumption of sugar with alcohol concentration of 16%, which is near to the theoretical value (Table 8). These results showed that the optimum condition seems to be addition of a small amount of oxygen only during the period in which pyruvic acid is formed. Similar finding was reported by Silveira et al. [33] who reported ethanol yield close to its theoretical value by the yeast *Kluyveromyces marxianus* UFV-3 under high lactose concentration and low oxygen level at the beginning of fermentation.

Table 7 – Effect of air at level of 150 $dm^3\,min^{-1}\,m^3$ of reactor volume during the whole period of fermentation on production of ethanol from 35% sucrose medium by *Saccharomyces cerevisiae*.

Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1}	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
0.0	2	350	0.00
12	46	220	1
24	102	144	1.1
36	132	71	2.5
48	172	0.00	1.6
60	132	0.00	0.0
72	120	0.00	0.0

Each value is the mean of three replicates.

Table 8 – Effect of air at level of 150 $dm^3\,min^{-1}\,m^3$ of reactor volume during the first 12 h of fermentation on production of ethanol from 35% sucrose medium by *Saccharomyces cerevisiae*.

Fermentation time (h)	Yeast cell number ($\times 10^6$ cfu ml^{-1}	Residual sugar ($g\,l^{-1}$)	Alcohol concentration (%)
0.0	2	350	0.00
12	44	212	2.9
24	96	138	7.2
36	100	98	11
48	121	0.0	16
60	98	0.0	16
72	67	0.0	10

Each value is the mean of three replicates.

4. Conclusion

A strain of *S. cerevisiae*, which showed marked fermentation activity, high ethanol and temperature tolerance and very good flocculation ability, was selected. Using this strain a set of fermentation experiments was allowed to go on with increasing the carbohydrate (sucrose) concentration. The results showed a stuck or sluggish fermentation at sucrose concentration of 25%. This means that the value corresponding to the substrate inhibition for the yeast strain under test was 25% sucrose concentration. Most of the substrate was immediately converted to ethanol, as a result of increasing the inoculum size from 3% to 6%. The ratio of added nitrogen to sucrose ($mg\,g^{-1}$), which gave the best results in this work was found to be 5 mg nitrogen as ammonium sulfate/g of sugar. For fermentation of certain sugar concentrations, we suggest that this ratio should be nearly constant. Supplementation of sucrose media with yeast extract ($6\,g\,l^{-1}$) together with thiamine ($0.2\,g\,l^{-1}$), greatly directed the fermentation pathway to production of ethanol with complete utilization of the sugar and great increase in the viable yeast cell numbers. The results showed that oxygen should be added only during the period in which pyruvic acid is formed. Accordingly we added a small amount of air ($150\,dm^3\,min^{-1}\,m^3$ of reactor volume) only during the first 12 h of fermentation. The results of this study showed a complete consumption of sugar with alcohol concentration of 16%, which was near to the theoretical value.

Acknowledgement

The author would like to thank the Alexander von Humboldt Foundation, Boon, Germany for sending the used fermenter as a donation.

REFERENCES

[1] Limtong S, Sringiew C, Yongmanitchai W. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresource Technol 2007;98:3367–74.

[2] Jones AM, Ingledew WM. Fuel ethanol production: appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. Process Biochem 1994;29:483–8.

[3] Dombek KM, Ingram LO. Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. Appl Environ Microbiol 1986;52:975–81.

[4] Bisson LV, Butzke CE. Diagnosis and rectification of stuck and sluggish fermentation. Am J Enol Viticulture 2000;51:168–77.

[5] Bely M, Rinaldi A, Dubourdieu D. Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. J Biosci Bioeng 2003;96:507–12.

[6] Beltran G, Esteve Zarzoso B, Rozes N, Mas A, Guillamon JM. Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentations kinetics and nitrogen consumption. J Agric Food Chem 2005; 53:996–1002.

[7] Ough CS, Crowell EA, Mooney LA. Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. Addition of amino acids, urea and ammonium: effects of fortification of intracellular and extracellular precursors. Am J Enol Viticulture 1988;39:243–9.

[8] Wang XD, Bohlscheid JC, Edwards CG. Fermentative activity and production of volatile compounds by *Saccharomyces* grown in synthetic grape juice media deficient in assimilable nitrogen and/or panthotenic acid. J Appl Microbiol 2003;94: 349–59.

[9] Berthels RR, Otero C, Bauer FF, Thevelein JM, Pretorius IS. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res 2004;4:683–9.

[10] McCaig R, McKee J, Pfisterer EA, Hysert DA, Munoz E, Ingledew WM. Very high gravity brewing—laboratory and pilot plant trials. J Am Soc Brew Chem 1992;50:18–26.

[11] Thomas KC, Ingledew WM. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mash. Appl Environ Microbiol 1990;56:2046–50.

[12] Bugajewska A, Wzorek W. Effect of selected activators and forms of yeasts on wine fermentation. Przemysł Fermentacyjny i Owocowo–Warzywny 1995;6:20–2 [Scientific-technical magazine for fermentation and fruit and vegetable industry].

[13] Bafrncova P, Smogrovi cová D, Sláviková I, Pátkoá J, Dömény Z. Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. Biotechnol Lett 1999;21:337–41.

[14] Kaczkowski J. Basis of biochemistry. Warsaw: WNT; 1993. p. 294.

[15] Kotarska K, Czupryński B, Kłosowski G. Effect of various activators on the course of alcoholic fermentation. J Food Eng 2006;77:965–71.

[16] Dragone G, Daniel P, João B. Factors influencing ethanol production rates at high-gravity brewing. Lebensm Wiss Technol 2004;37:797–802.

[17] Luparia V, Soubeyrand V, Berges T, Julien A, Salmon JM. Assimilation of grape phytosterols by *Saccharomyces cerevisiae* and their impact on enological fermentations. Appl Microbiol Biotechnol 2004;65:25–32.

[18] Munroe J. Fermentation. In: Hardwick WA, editor. Handbook of brewing. New York: Marcel Dekker Inc; 1994. p. 323–53.

[19] Banat IM, Nigam P, Marchant R. Isolation of thermotolerant, fermentative yeasts growing at 52 °C and producing ethanol at 45 °C and 50 °C. World J Microbiol Biotechnol 1992;8: 259–63.

[20] Banat IM, Marchant R. Characterization and potential industrial applications of five novel thermotolerant, fermentative yeasts strains. World J Microbiol Biotechnol 1995;11:304–6.

[21] Bai FW, Chen LJ, Anderson WA, Moo-Young M. Parameter oscillations in very high gravity medium continuous ethanol fermentation and their attenuation on multi-stage packed column bioreactor system. Biotechnol Bioeng 2008;88:558–66.
[22] Kourkoutas Y, Bekatorou A, Banat IM, Marchant R, Koutinas AA. Immobilization technologies and supporting materials suitable in alcohol beverages production: a review. Food Microbiol 2004;21:377–97.
[23] Jin YL, Speers RA. Flocculation of *Saccharomyces cerevisiae*. Food Res Int 1998;31:421–40.
[24] Sharma SK, Kalra KL, Grewal HS. Fermentation of enzymatically saccharified sunflower stalks for ethanol production and its scale up. Bioresource Technol 2002;85:31–3.
[25] Kahlon SS, Kumar P. Simulation of fermentation conditions for ethanol production from water hyacinth. Ind J Ecol 1987;14:213–7.
[26] Taillandier P, Portugal FR, Fuster A, Strehaiano P. Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. Food Microbiol 2007;24:95–100.
[27] Thomas KC, Hynes SH, Ingledew WM. Effect of nitrogen limitation on synthesis of enzymes in *Saccharomyces cerevisiae* during fermentation of high concentration of carbohydrates. Biotechnol Lett 1996;18:1165–8.
[28] Alfenore S, Molina-Jouve C, Guillouet S, Uribelarrea JL, Goma G, Benbadis L. Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process. Appl Microbiol Biotechnol 2002;60:67–72.
[29] Salik FLM, Povoh NP. Método espectrofotométrico para determinação de teores alcoólicos em misturas hidroalcoólicas. Congresso Nacional da STAB. 5, Águas de São Pedro, STAB, Anais, Piracicaba; 1993. p. 262–6.
[30] Herbert D, Phipps P, Strange R. Chemical analysis of microbial cells. Meth Microbiol 1977;5B:209–345.
[31] You KM, Rosenfield CL, Knipple DC. Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl Environ Microbiol 2003;69:1499–503.
[32] Shang F, Wen S, Wang X, Tan T. High-cell-density fermentation for ergosterol production by *Saccharomyces cerevisiae*. J Biosci Bioeng 2006;101:38–41.
[33] Silveira WB, Passos FJ, Mantovani HC, Passos FM. Ethanol production from cheese whey permeate by *Kluyveromyces marxianus* UFV-3: a flux analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen levels. Enz Microb Technol 2005;36:930–6.