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Ethanol Production Kinetics by a Thermo-Tolerant Mutant of *Saccharomyces Cerevisiae* from Starch Industry Waste (Hydrol)

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Abstract

A thermo-tolerant and deoxyglucose-resistant mutant of *Saccharomyces cerevisiae* was developed and employed to convert them to fuel ethanol in a 150 litre fermenter. Maximum ethanol production was achieved when fermentation of dextrozyme- treated hydrol was carried out for about 36 hours under optimized fermenting conditions. The maximum specific ethanol production rate (q_P), and overall ethanol yield ($Y_{P/S}$) were found to be 2.82 g L^{-1} h^{-1} and 0.49 g/g respectively. Determination of activation energy for cell growth (E_a= 20.8 kJ/mol) and death (E_d = 19.1 kJ/mol) and product formation and inactivation (E_P=35.8 kJ/mol and E_{dp} = 33.5 kJ/mol) revealed the thermo-stability of the organism for up to 47°C.

Keywords: Corn steep liquor; Hydrol; Temperature; Specific productivity; Product yield.

Introduction

Due to increasing environmental and political pressures, depletion of fossil fuel reserves and unstable petrol prices have increased interests towards development of the ethanol from biomass at industrial scale. Starch industry releases hydrol, a waste material which is an environmental pollutant but rich in fermentable carbohydrates. Its discharge in public sewerage system will support the growth of numerous pathogenic organisms which will cause health hazards.Sugar cane juice, sugar beet and molasses are among the most important raw materials for alcohol production, using *Saccharomyces cerevisiae*. Increased cost of molasses has forced the distilleries to seek other feed stocks for ethanol production in Pakistan. Enzose hydrol, a byproduct from starch industry contains 56, 12, 5 and 5 % glucose, maltose, maltotriose, and oligosaccharides respectively and is a good and cheap source of fermentable sugars. Maltotriose and oligo-saccharides are not completely consumed by *S. cerevisiae* [1] therefore, their pre-hydrolysis with dextrozyme is essential for complete conversion into ethanol [2]. Temperature in temperate countries goes beyond 40°C in summer and there are high energetic costs linked to fermentation regulation in distilleries. Thus, there is a dire need to use thermo-tolerant yeasts [3] which grow in the temperature range of 40-50°C. Temperature has direct effect on the solubility of O_2 and CO_2 dissolved in the culture medium, and the rate of oxygen transfer within the culture system, thus effecting growth and product formation.

The influences of temperatures on ethanol fermentation by *S. cerevisiae* with regard to the kinetic parameters related to biomass and ethanol production and sugar consumption can be quantified in time course study to predict growth like biomass yields, and maximum growth rate,

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understanding metabolic networks and their regulation [4]. Application of such data permits engineers to optimize product yields and calculate the energy requirements of a process in industrial bioreactors [5]. Although the production of ethanol from Enzose concentrates in shake flasks and small fermenters has been well studied [6, 7], its scale-up production in pilot plant has greater applications.

The main purpose of this research work was to develop an effective fermentation process for scaling-up production of ethanol by a thermotolerant mutant strain of *S. cerevisiae* M-9 in 150-l fully controlled fermenter after studying dynamic behaviour of *S. cerevisiae* during fermentation process in 23-l fermenter.

Experimental

Chemicals

All the chemicals were purchased from Sigma Chemical Company and were of analytical grade. For saccharification of oligosaccahrides, maltose and maltotriose, dextrozyme (Novozymes) was applied. It contains an improved glucoamylase (EC 3.2.1.3) for faster hydrolysis and a pullulanase that gives extremely high performance. For applying pre-saccharification, the medium was heated to 60°C and enzyme (1.0 IU/g maltose, maltotriose and oligosaccharides) was added. The temperature was maintained for 1 h, and then cooling was programmed to end at 20-50°C (as needed) when the appropriate amount of pre-culture was added.

Substrate

Enzose hydrol or corn molasses (obtained during the washing of dextrose) was purchased from CPC Rafhan Maize Product Co., Faisalabad. It was analyzed for total solids, total sugars, ash, and protein. It was diluted with distilled water to required concentration of sugars.

Microorganism and cultural conditions

Saccharomyces cerevisiae (SAF culture, France) was maintained on yeast extract, malt extract, peptone, glucose and agar slants at 10°C. To prepare seed culture of *S. cerevisiae*, single colonies were used to inoculate I-l conical flasks containing 250 ml medium which contained in $g.l^{-1}$: glucose, 10; $(NH_4)_2$ HPO_4, 0.64 , and yeast extract 2.5; at pH 5.5 and incubated for 18 h on an orbital shaker at 150 rpm at 30°C. The culture was exposed to γ-rays of 800 kG and enriched at 45°C in 17 % total reducing sugars (TRS) in molasses + 1.5 % deoxyglucose, to attain, thermo-tolerant as well as deoxyglucose-resistant strain of *S. cerevisiae*. After 4–d growth, plate-screening [8, 9] gave us one variant which was designated *S. cerevisiae* M-9.

Procedure

Fermentation studies were carried out in a micro-processor controlled 23-l stainless steel fermenter (Biostat C5, Braun Biotechnology, Melsungen, Germany) 15-l working-volume vessel equipped with instruments and controllers for parameters such as agitation, temperature, pH, and dissolved oxygen and fitted with a reflux cooler in the gas exhaust to minimize evaporation. The vessel was filled with medium containing hydrol (15 % TRS) and the following and corn steep liquor (25 g/l) found optimum. The pH was adjusted to 5.5 (optimum) and the medium was steam-sterilized *in situ* for 15 min. The fermenter was inoculated with 10 % (v/v) active inoculum. The aeration was carried out through a sparger at 15 l/min for 8 h to enhance biomass production before switching over to 3 l/min. This process lasted up to 36 h during which foaming was controlled by adding silicone oil as an antifoaming agent. Duplicate fermentations were carried out in all studies. Temperature-dependent formation of ethanol occurred along with minute quantities of acetic acid, succinic acid and glycerol. pH was found to have dropped due to formation of acetic acid, it was controlled automatically at 5.5 using KOH by a digitally controlled accessories of the bioreactor.

Analytical methods

Cell growth

The amount of growth was measured gravimetrically as dry cell mass after centrifugation of a portion of the cells (100 ml) (5,000 g at 10 °C for 10 min) and suspension in physiological saline.

Determination of ethanol and unfermented sugars

Cell-free supernatant was used for determining ethanol and unfermented sugars. Total sugars were determined using a Brix hydrometer measuring specific gravity (Atago, ATC-1, Brix:0-32%, Japan). Ethanol was determined gravimetrically through laboratory-scale distillation of fixed volume of fermented broth. Alternatively glucose, maltose, maltotriose, oligosaccharides acetic acid, succinin acid, glycerol and ethanol were analysed by HPLC (Perkin Elmer, USA) using column HPX-87H (300 x 78 mm) (Bio, Richmond, California) maintained at 45°C in a column oven. Sulphuric acid (0.001 N) in HPLC grade water was used as a mobile phase at 0.6 $ml.min^{-1}$. The samples were detected by refractive index detector and quantified using Turbochron 4 software of Perkin Elmer, USA.

Determination of kinetic parameters

All kinetic parameters for batch fermentation was calculated as described previously [10]. Empirical approach of the Arrhenius [11] was used to describe the relationship of temperature dependent growth and product formation for a temperature range 20-50°C. For this purpose, specific rate of growth (μ) and product formation (q_p, g/g cells. h) were used to calculate the demand of activation energy for cell growth and death, product formation and product inactivation.

Results and Discussion

To avoid the inhibitory effects of substrate and ethanol, 15% total reducing sugars (TRS) in hydrol were used in optimization studies. Studies on dextrozyme pretreatment of hydrol in shake flask and fermentor studies concluded that one IU Dextrozyme/g tri-saccharides and oligosaccharides were sufficient to get 100 % glucose and produce ethanol. Among nitrogen sources (ammonium sulphate, urea and corn steep liquor), corn steep liquor (25 g/l) supported maximum ethanol production (74 g/l) and was used as only additive to hydrol for ethanol production. It was observed that maximum values of ethanol (99 g/l) were obtained with 1 vvm aeration rate for 8 hours, followed by 0.25 vvm for another 28 hours at 40°C at 250 rmp agitation speed in 23-litre fermentor, following growth on 150 g TRS/l.

The fermentative capacity of the mutant strain was strongly affected by the temperature of fermentation. A significant ($p \leq 0.05$) difference among the specific rates of ethanol production (q_p), ethanol yield, specific ethanol yield and volumetric rate of ethanol with temperature is presented in the Table 1. Its effect on specific growth rate (μ), cell mass formation rate, substrate consumption rate and cell yield is shown in Table 2. Thus effect of temperature on all kinetic parameters was evident from the high values of *F* (Table 1 and Table 2), which signified that influence of temperature on all kinetic parameters was highly significant.

Table 1. **Kinetic parameters of *S. cerevisiae* M -9 for ethanol production following growth on 15 % sugars in Dextrozyme-pretreated hydrol in a fully controlled 23-l fermenter.**

Temperature ° C	Q_P (g/ lh)	$Y_{P/S}$ (g/g substrate)	$Y_{P/X}$ (g/g cells	q_P (g/g h)
20	1.3g	0.27e	4.30c	0.66d
25	1.6f	0.31d	6.70ab	1.47c
30	2.2d	0.47b	7.80ab	1.69b
35	2.6b	0.48b	8.00ab	2.08a
40	2.8a	0.50a	8.25a	2.10a
45	2.5c	0.40c	7.10ab	1.49c
47	1.8e	0.32d	6.50b	0.62d
50	0.9h	0.15f	3.7c	0.41e
F	356.1	239.4	10.0	356.1
P	0.0000	0.0000	0.0002	0.0000

Each value is a mean of two experiments. Standard deviation among replicates varied between 3.5 to 5.0 % of mean values and has not been presented. Values followed by different letters differ significantly at $p \leq 0.05$.

Table 2 **Temperature effect on biomass formation and substrate consumption by *S. cerevisiae* M-9 during ethanol production following growth on 15 % sugars in Dextrozyme-pretreated hydrol in a fully controlled 23-l fermenter.**

Temperature ° C	Q_X (g/ lh)	$Y_{X/S}$ (g/g)	Q_S (g/lh)	μ (1/h)
20	0.29e	0.071bc	2.6g	0.14e
25	0.46c	0.080ab	4.0e	0.20d
30	0.56b	0.084ab	4.7c	0.21c
35	0.60a	0.088a	4.8b	0.24b
40	0.61a	0.091a	5.0a	0.26a
45	0.46c	0.078a	4.3d	0.21c
47	0.43d	0.056c	3.9f	0.19d
50	0.24f	0.035d	2.1h	0.11f
F	257.4	276.9	290.8	140.02
P	0.000	0.0000	0.000	0.000

Standard deviation among replicates varied between 3.5 to 5.5 % of mean values and has not been presented. Values followed by different letters differ significantly at $p \leq 0.05$. *F* and *P* values indicated that effect of temperature on all parameters was highly significant.

The maximum specific growth rate, specific death rate of cells and the maximum product formation rate of cells increased as the temperature increased up to 40°C. Expressed by the Arrhenius relationship, the temperature dependence of these parameters is shown in (Fig. 1a and Fig. 1b). The estimated values of activation energies for cell growth (20.8 kJ/mol)), ethanol formation (35.8 kJ /mol) and for cell death (19.1 kJ/mol) and product inactivation (33.5kJ/mol) were found contrary to the higher values reported in literature for mesophilic organisms (138.9-177 kJ/ mol) [12]. The magnitude of activation energy during deactivation phase was lower than corresponding magnitude in the growth phase and confirms the findings of others for thermophilic organisms [13-15]. Requirement of lower energy of activation (E_a) for growth and product formation is considered indicator of thermostable metabolic network [15]. These stabilizing forces were supposedly provided by the system itself during product formation, most probably by acquiring chaperones, which assisted the folding of protein within cells [16]. Other authors also reported that in stressed conditions *S. cerevisiae* accumulated glycerol, or erythretol under temperature stress [17-18].

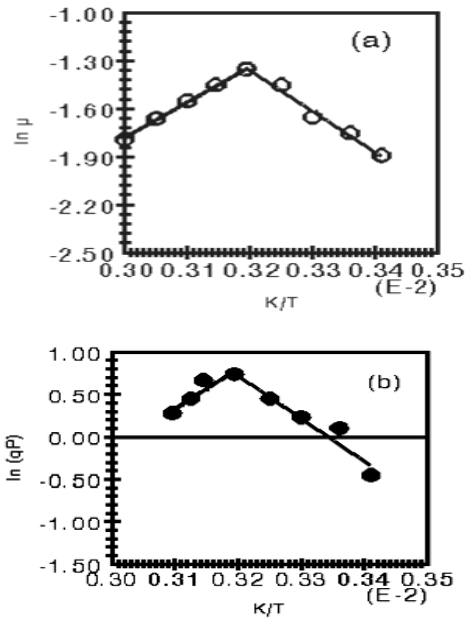

Figure 1. **Determination of activation energy for growth (a) and product formation (b) using Arrhenius relationship.**

Effect of substrate concentration on kinetic parameters (Fig. 2) revealed that the sugar concentration of 15% gave highest values of all kinetic parameters except $Q_{P,\ which}$ was maximum on 20% TRS in hydrol. It was observed that high sugar was accompanied by increased glycerol, citric acid and acetic acid production. Low sugar concentration ultimately resulted in increase of ethanol production in the medium. Therefore optimized sugar concentration necessary for ethanol production was 15% (W/V).

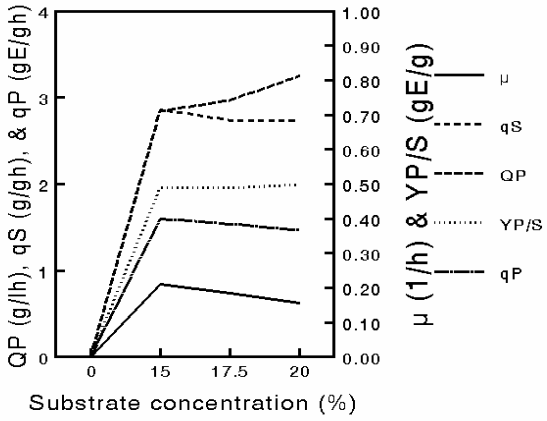

Figure 2. **Effect of substrate concentration on specific growth rate (μ), specific substrate consumption (q_S), volumetric rate of product formation (Q_P), product yield ($Y_{P/S}$) and specific productivity (q_P) in 23-l fermenter using Dextrozyme-pretreated hydrol as substrate, and CSL (25 g/l) as nitrogen source. Initial flow rate was 1 vv for 8 h followed by 0.25 vvm in agitated vessel (250 rpm) at 40 °C.**

Time course production under optimized conditions in 150-litre fermentor

The incubation time for achieving maximum product level is governed by the characteristics of the culture in small and big fermenter and is based on growth rate and product production during time course study. The time course of product and cell mass formation by the yeast from hydrol, as shown in Fig. 3, indicated that organism produced high titres of alcohol at 24 h of incubation in the log phase. Representative growth curves of the *Saccharomyces cerevisiae* M-9 (Fig. 3) showed that in all cases the cell grew exponentially for the first 8-16 hours of the SSF process and then there was a short deceleration phase and finally a stationary phase. It was noteworthy that before stationary phase, more than half of the total saccharides were consumed and more than 50% of the total ethanol was produced. Though ethanol is a primary metabolite but its formation was both growth- and non-growth-associated.

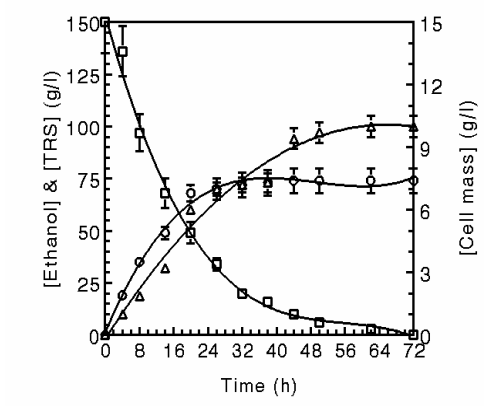

Figure 3. **Representative time course of ethanol (○), cell mass production (Δ) and TRS (□) in fermenter from 15 % hydrol supplemented with 25 g/l corn steep liquor in 150-l fully controlled fermenter. Each reading is a mean of two observations. Error bars show standard deviation between them.**

Conclusion

It was concluded that a percentage of 7.8 to 8.6 increase in the ethanol production was achieved under the optimized fermentation conditions, as compared with the basal medium used as control. Although the results of these investigations are based on experiments performed in 150- litre fermenter, they provide valuable information for the production of this product in industrial production process.

At the end of alcoholic fermentation, a significant amount of residual cells is attained, which in turn, could become a source for several products of economic interest (yeast extract, enzymes, nucleic acid, among others [19]. This study may provide a better understanding of temperature effects on the cell activities for further development of the processes for maximizing ethanol production in vinification and use in petrol blending.

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