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Hyper Production of Ethanol from Cane Molasses at Optimized Agitational Intensity Using Indigenous Thermotolerant *Kluyveromyces Marxianus*

[1]Shaheen Aziz, [1]Farman Ali Shah, [2]Hafeez Ur Rehman Memon, [1]Suhail Ahmed Soomro, [1]Aziza Bano and [3]Muhammad Ibrahim Rajoka

[1]Department of Chemical Engineering, Mehran University of Engineering and Technology, Jamshoro, Sindh, Pakistan.
[2]Institute of Petroleum and Natural Gas Engineering, Mehran University of Engineering and Technology, Jamshoro, Sindh, Pakistan
[3]Government College University, Faisalabad, Pakistan

Abstract: The wild and M15 mutant (γ-ray induced) *Kluyveromyces marxianus* yeast were used for the current study. The two strains of yeasts were used for the hyper-production of ethanol. The fermentation was carried out in microprocessor controlled 23-L stainless steel fermenter. Initially, the production of ethanol was optimized by using the two strains of *K. marxianus*. The ethanol production using, wild and mutant organism were optimized at temperature of 40 ˚C, initial pH 5.5, molasses (15% sugars) and nitrogen Source, ammonium sulphate (0.75%). The effect of various agitation rates (250-450 rpm) on production of ethanol by *K. marxianus* cells was studied at optimized conditions. It was observed that, at 300rpm, maximum (7.5%) ethanol was produced, based on 15% sugar. Further increase in agitation intensity did not increase ethanol production in both strains of *K. marxianus*.

Key words: Kluyveromyces Marxianus, Ethanol, Molasses, Agitational Intensity, Bioreactor

INTRODUCTION

The biofuels policy aims to promote the use of ethanol made from biomass in transport vehicles, to provide economic opportunities for people in rural areas in developing countries. The central policy of bio-fuel is jobs creation, greater efficiency in the general business, and protection of the environment. Use of indigenous renewable sources of energy have potential to provide energy services with zero or almost zero emissions of both air pollutants and green house gases (Ayhan, 2009). Additionally, increasing the growth temperature usually results in the biosynthesis of heat-shock proteins which are implicated in conferring thermal and ethanol cross-tolerance in various organisms (Abdel-Fattah *et al.* 2000). Molasses having high sucrose was unfavourable outcome on the final produced ethanol with cheap activity of invertase or fructofuranosidase (Gough *et al.* 1996).

Present study pertaining with the production of ethanol from thermotolerant yeast of *Kluyveromyces marxianus*, which was first described in 1888 by EC. Hansen. At that time, it was named *Saccharomyces marxianus* after Marxianus, the person who originally isolated this yeast from grapes (Gustavo *et al.* 2008). In Pakistan, only *Saccharomyces cerevisiae* is being used for the production of ethanol Shah, (2010) and there is no use of theromotolerant *Kluyveromyces marxianus*. Whereas in India and Brazil, *K. marxianus* strains are commonaly used for the production of ethanol. Indigenous *Kluyveromyces marxianus* yeast will be best economical source for the fermentation industries in Pakistan. Ethanol production in tropical countries at high temperature is a key requirement for maximum production, where average day-time temperatures are high throughout the year. Other benifits are fast process of fermentation at high temperature are not only reduced risk of contamination but also decreased in cooling costs (Limtong *et al.* 2007).

MATERIALS AND METHODS

The experimental work was carried out in collaboration with National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan**.** All chemicals were purchased from Sigma/Aldrich Chemical Co., Germany. Molasses was donated by Habib Sugars Mills (PVT), Nawabshah and Crescent Sugar Mills, Faisalabad.

Corresponding Author: Shaheen Aziz, Department of Chemical Engineering, Mehran University of Engineering and Technology, Jamshoro, Sindh, Pakistan.
E-mail: Shaheen_aziz1@yahoo.com

This strain (D-67283) was collected from Shakkar Gunj Sugar Mills, Jhang, selected through Gamma rays on 1.5 % (w/v) deoxy-Dglucose (DG) in liquid medium after growth at 60 °C for 5 days and designated as *K. marxianus* M15 as shown in Figure 1.

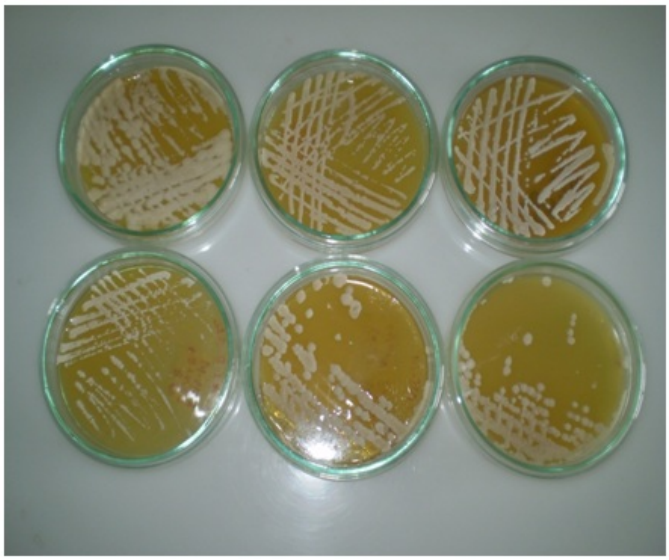

Fig. 1: Pure culture of thermotolerant *Klyveromyces marxianus* yeast

Inoculum Preparation

For inocula preparation the yeast medium was used having the following composition (w/v):

Components	(%)
Peptone	0.5
Yeast extract	1.0
NaCl	0.5
Glucose	2.0
pH	5.5

All chemicals were weighed and mixed in distilled water one by one to prevent possible precipitation in a conical flask. The pH of the media was adjusted with 1M HCl/1M NaOH and volume was made (50 ml/flask) with distilled water. The flasks were plugged with cotton, covered with aluminum foil and autoclaved at 121 °C and 1.05 kg/cm^2 pressure for 15 min. After cooling to ambient temperature, 2ml of sterilized 50% (w/v) stock solution of glucose were aseptically added to the autoclaved yeast medium as a carbon source. The test flasks containing autoclaved yeast media were inoculated with a loop- full of *Kluyveromyces marxianus* under aseptic conditions. They were allowed to grow at 30±2 ºC (unless mentioned otherwise) on a rotary shaker (120 rpm) for 24 h. The cells were harvested and centrifuged at 10,000 rpm. (8331×g) for 15 min, washed in saline solution containing 0.01% yeast extract. The biomass content was estimated on the basis of absorptionmetry (cells turbidity) by measurement of the light transmitted at 610 nm. This population was used for mutational work and to inoculate the growth media.

Fermentor 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 sugars (15 % TRS) in molasses supplemented with optimum concentration of $(NH_4)_2 SO_4$ (7.5 g L^{-1}) or other carbon sources as given above. The pH was adjusted to 5.5 (optimum) and the medium was steam-sterilized in situ for 30 min. The fermenter was inoculated with 10 % (v/v) active inoculum. The aeration was carried out through a Sparger at 15 L min^{-1} for 8 h to enhance biomass production before switching over to 3 L min^{-1}. This process lasted up to 36 h during which foaming was controlled by adding silicone oil as an antifoaming agent. Substrate, nitrogen source, pH and temperature-dependent formation of ethanol occurred along with minute quantities of acetic acid, succinic acid and glycerol. pH dropped due to formation of acetic acid, which was controlled automatically at 5.5 using KOH. In all studies, 100 ml samples in triplicate were collected from both parental and mutant cultures after every 8 h for different Analysis.

Determination of Growth Kinetic Parameters:

Fermentation kinetic parameter of K.marxianus Wild (W) and its mutant (M) strain for growth substrate utilization and product formation were calculated (Aiba, etal.1973). Following formulae were used for the parameters:

Yp/x [Product yield coefficient with respect to cell mass] = dP/dx
Yp/s [Product yield coefficient with respect to substrate] = dP/ds
qp [Product yield coefficient with respect to substrate] = μ x Yp/x
qs [Substrate utilization] = μ x /Yx/s

If the value of μ is constant, then above equation represents the so-called exponential growth, where growth is proportional to the mass of the cells present. Note that the specific growth rate is related to the mass doubling time, t_d by

$$t_d = \frac{0.693}{\mu}.$$

Other volumetric rates Q_x, Q_s and Q_p in g L^{-1} h^{-1} were calculated by graphs using M-Stat software by using the values of cell mass formation, substrate consumption and product formation.

Statistical Analysis:

The effect of treatments was compared by the protected least significant difference method is presented in the form of probability (p) using Duncan multiple range test with Mstat C software.

RESULTS AND DISCUSSION

The effect of different agitation rates (250 - 450 rpm) on production of ethanol by *K. marxianus cells* was carried out (Table 1&2). The maximum amount of ethanol produced on the basis of sugar used was more than 89.96 %. Further increase in agitation intensity did not increase ethanol production in both organisms. Hence, agitation rate of 300 rpm was optimised. Thus an increase in ethanol production on increasing agitation could be due to increased oxygen transfer rate, increased surface area of contact with the media components or better dispersability of the substrate. There was no enhancement in ethanol production on further increasing the agitation rates. However at agitation rates of 300 rpm, there was a reduction in growth as well as ethanol production due to shearing stress on the organism.

The production of ethanol is an anaerobic fermentation process. Thus supply of oxygen to the yeast culture is of great importance as it is needed to support an initial amount of cell mass for maximum ethanol production. At a constant rate of oxygen supply, agitation rate supports uniform distribution of cells and maintain a constant temperature by uniformly stirring the media to dissipate excess heat.

The maximum amount of ethanol (75 g L^{-1}) produced when the aeration rate was kept at 0.30 LL $^{-1}$min^{-1}. The sugar consumption, dry mycelial weight and final pH of the medium were 149.0g L^{-1}, 9.5 g L^{-1} and 5.5, respectively optimized for ethanol and FFase production using temperature at 45 ºC as shown in Figure. 2.

Table 1: Fermentation kinetic parameters of K. marxianus (W) and its mutant strain (M) for growth and substrate utilization using ammonium sulphate (0.75%) , pH (5.5) added in molasses medium (15% sugars) on different imitational intensity(RPM) at 40°C under control conditions.

RPM	Strain	μ (h^{-1})	Q_x (g cell/l/h)	$Y_{x/s}$ (g/l/h)	Q_s (g/l/h)	q_s (g/g/ h)	t_d (h)
250	W	0.24^{cde}	0.855^{a}	0.082^{a}	3.210^{d}	2.92^{bcd}	2.88^{d}
	M	0.30^{ab}	0.890^{a}	0.091^{a}	4.910^{a}	3.29^{ab}	2.31^{f}
300	W	0.29^{bc}	0.850^{a}	0.090^{a}	2.452^{e}	3.22^{abc}	2.39^{f}
	M	0.35^{a}	0.895^{a}	0.099^{a}	5.012^{a}	3.53^{a}	1.98^{g}
350	W	0.21^{def}	0.830^{a}	0.076^{a}	3.192^{d}	2.76^{cd}	3.30^{c}
	M	0.27^{bc}	0.874^{a}	0.085^{a}	4.815^{ab}	3.18^{abc}	2.57^{e}
400	W	0.20^{ef}	0.822^{a}	0.072^{a}	2.340^{e}	2.77^{cd}	3.46^{b}
	M	0.26^{bcd}	0.854^{a}	0.082^{a}	4.101^{c}	3.18^{abc}	2.66^{e}
450	W	0.18^{f}	0.757^{a}	0.070^{a}	2.250^{e}	2.57^{d}	3.85^{a}
	M	0.24^{cde}	0.810^{a}	0.080^{a}	4.250^{bc}	3.00^{bcd}	2.88^{d}
LSDvalues($P \leq 0.05$)		0.05386	0.1425	0.05386	0.6235	0.5138	0.09329

Table 2: Fermentation kinetic parameters of K. marxianus (W) and its mutant strain (M) for growth and product formation using ammonium sulphate (0.75%) , pH (5.5) added in molasses medium (15% sugars) on different Agitational intensity(RPM) at 40°C under control conditions.

RPM	Strain	Q_p (g/l/h)	$Y_{p/s}$ (g/g subs)	$Y_{p/x}$ (IU/gcells)	q_p (g/g/ h)
250	W	4.85^{abc}	0.44^{bc}	5.70^{abc}	1.37^{d}
	M	5.12^{a}	0.48^{ab}	6.13^{ab}	1.84^{b}
300	W	4.90^{ab}	0.46^{abc}	5.80^{abc}	1.68^{bc}
	M	5.36^{a}	0.51^{a}	6.25^{a}	2.18^{a}
350	W	4.05^{d}	0.42^{bc}	5.20^{c}	1.09^{ef}
	M	4.52^{bcd}	0.46^{abc}	5.39^{abc}	1.45^{cd}
400	W	4.01^{d}	0.40^{bc}	5.10^{bc}	1.02^{ef}
	M	4.35^{bcd}	0.43^{bc}	5.27^{bc}	1.37^{d}
450	W	3.77^{d}	0.41^{c}	5.02^{c}	0.90^{f}
	M	4.12^{cd}	0.42^{bc}	5.10^{bc}	1.22^{de}
LSD values ($P \leq 0.05$)		0.7463	0.07617	0.9140	0.2526

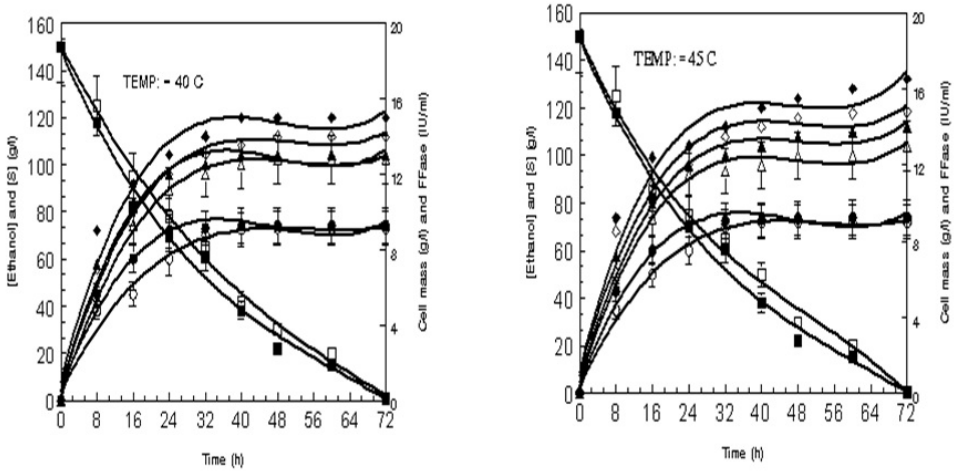

Fig. 2: Representative time of ethanol and FFase production in molasses medium supplemented with ammonium sulphate (0.75 g/l) for wild at 40 °C and for M15 at 45 °C respectively. All other variables were kept constant while pH of the medium was varied.

The concentration of dissolved oxygen in a suspension of respiring microorganisms usually depends on the rate of oxygen transfer from the gas phase to the liquid, on the rate at which oxygen is transported to the site of utilization, and on the rate of its consumption by the microorganism, as mentioned earlier.

Agitational intensity of 250 to 450 rpm was tested to see its impact on ethanol production (Table 1&2). It was found that 300 rpm is the best one (The maximum amount of ethanol 75 g L^{-1} was produced when the aeration rate was kept at 0.30 L $L^{-1}min^{-1}$. The sugar consumption, dry mycelial weight and final pH of the medium were 149.0g L^{-1} 9.5 g L^{-1} and 5.5, respectively. However, the literature search revealed that the highest agitational intensity of 700 rpm was used by Oniscu *et al.* (2004) at smaller volumes of 5 liters for the fermentation. More agitation produces more gas dispersion, and more gas dispersion produces more mass transfer. This combination gives higher mass transfer and supports higher productivity.

In fermentation process, In order to perform a given bioconversion in a fermenter, mass and heat transfer, gas dispersion, and a certain homogenization are required. These factors are generally achieved by agitation with an impeller (Yoshida *et al.* 2005). But in fungal fermentation, agitation not only fulfills the above functions, but as a side effect may also influence the morphology of fungi. Strong influence on the physical properties due to the morphology of the organisms as fungal cultivation broth which causes various problems in industrial fermenters with respect to mass dispersion, heat transfer, gas dispersion and homogenization. (Braun and Vecht-Lifshitz, 1991). The process was carried out at 35 °C, pH 5.8 and 200 rpm stirring speed for 4 days (Suresh *et al.* 2000).

Conclusion:

Most effective stirring speed in all experiments was 300 rpm and oxygen flow rate was 1.0 vvm for 8 h followed by 0.1 vvm for 28- 32 h. The mutant supported maximum ethanol and invertase at 45°C. Other optimized fermentation conditions as discused earlier did not differ significantly and were maintained in all

other experiments. Both wild (W) and mutant (M) strains of *Kluyveromyces marxianus* showed same optimum pH = 5.5. However, wild organism showed maximum specific growth rate at 40 °C while mutant organism showed maximum specific growth rate and ethanol formation rate at 45 °C under controlled temperature. The ethanol production of mutant was improved 1.4 fold as compared to parental (wild) strain.

Mutant-derivative (M15) was stronger over its parental culture due to its stable metabolic activity, which showed more thermal stability and production of ethanol at 50-65 °C at which wild organism could not grow.

The ethanol production process can be used as good model system for investigation of molecular basis of thermostability of moderately thermostable network. The results presented for the ethanol production at 23 L fermenter level is considerably significant for further development of a large scale production process, using mutant derivative. The large scale production may also be anticipated as economically feasible as the optimized carbon and nitrogen sources are cheap and found in abundance in agricultural countries, like Pakistan. This will also support industrial scale production proposal.

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