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Production of Ethanol by Indigenous Wild and Mutant Strain of Thermotolerant *Kluyveromyces Marxianus* Under Optimized Fermentation Conditions

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Abstract

The maximum ethanol production and β-fructofuranocidase formation under fermentation studies were carried out in microprocessor controlled 23-L stainless steel fermenter at the following conditions: Temp = 40 ˚C (wild &mutant organism) , pH= 5.5, carbon source (molasses=15% sugar) nitrogen source (ammonium sulphate 0.75%), 300 RPM stirring speed and oxygen flow rate was 0.1 vvm. Mutant. Strain of thermotolerant *Kluyveromyces marxianus* M15 produced maximum production of ethanol at 48hr.All Kinetic Parameters have been studied for the utilization of substrate and production of maximum ethanol for both wild and mutant strains. It has been observed that wild strain was growing up to 55^0C while the mutant strain was growing up to 65^0C. In this comparison study, wild and mutant strain showed that Mutant-derived M15 was stronger over its parental culture due to its more thermal stability and production of ethanol at 65 ^{o}C at which wild organism could not grow.

Keywords: Yeast, Fermentation, *Kluyveromyces marxianus,* metabolic network, thermal effects, Ethanol, Kinetic parameters.

Introduction

Highly thermotolerant growing yeast gives lot of benefits over all in the process of fermentation. It reduces contamination problems, cost of energy and environmental problems that becomes more feasible and cost effective due to the presence of strong enzyme in the yeast. The growth and ethanol production by indigenous strain of *Kluyveromyces marxianus* and *Sacchromyces cerevisiae* were compared under the same conditions. *K.marxianus* DMKU3-1042 was found to be most suitable strain for high-temperature growth and ethanol production at 45 °C [1]. Strains of *Kluyveromyces marxianus* have attracted much attention because they can grow at higher temperature and produced ethanol comparable to that by *S. cerevisiae* at industrial scale[2]. It is noteworthy for its high product formation rate, which would make it an attractive candidate for industrial ethanol production in summer when temperature goes to 45-50 °C in temperate regions of the world.

A number of groups have taken on the challenge of isolating more thermo-tolerant organisms for simultaneous saccharification and fermentation of biomass for ethanol production or for ethanol from molasses but production of ethanol using thermo-tolerant yeasts has not reached a level sufficient for commercial production at 45-50 °C because of instability of their invertases at 45-50 °C (unpublished results). Thus there is high energy costs linked to fermentation regulation in distilleries and necessitates the application of thermo-tolerant yeasts, which produce ethanol in the temperature range of 40-50 °C. Temperature has direct effect on the solubility of O_2 and

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CO_2 in the culture medium, and the rate of oxygen transfer within the culture system, thus effecting growth and product formation.

Here we present data on the production of ethanol from a newly developed indigenous thermophilic mutant derivative (M15) of *Kluyveromyces marxianus*, capable of growing in fully controlled bioreactor up to 65 °C. The influence of temperatures on ethanol production can be quantified in time course study to estimate maximum growth rate, and maximum specific product formation rate for understanding metabolic network and their regulation [3]. Application of such data permits engineers to optimize product yields, predict the hidden process regulatory mechanism and calculate the energy requirements of a process in industrial bioreactors [4, 5].

Materials and Methods

Growth of organism

Thermotolerant strain of *Kluyveromyces marxianus* D-67283 was collected from Shakkar Gunj Sugar Mills, Jhang at Faisalabad. The strain was maintained on Saboraud's agar plates and slants as described earlier [6].

Chemicals and growth media

All chemicals were purchased from Sigma Chemical Co., Missouri, USA. *K. marxianus* was grown in submerged formation ,which was carried out in 250 ml conical flasks containg 100ml of yeast in saboraud's media contained yeast extract (0.5 %) , peptone (0.5%), NaCl (0.5%) and glucose (2 %). The initial pH of the medium was adjusted to 5.5 with 1 M HCl. For other growth studies, the seed culture developed on glucose was used as inoculum and washed twice with sterile saline before use.

Isolation of mutants

K. marxianus cells were cultured in yeast culture medium at 40 °C for 24 h, centrifuged at 5,000 rpm for 15 min and suspended in 50 ml of saline containing 0.02% yeast extract. The cells of 3.0 absorbance (at 610 nm) were dispensed equally in 30 ml McCartney vials. The cells were exposed to different doses of γ-ray in a Co-60 irradiator. The exposure of cell suspension ($\approx 3.0 \times 10^9$ cells ml^{-1}) to γ-irradiation of 1200 Gy gave approximately a 3-log reduction in colony forming units. The radiated cells were allowed to express in the presence of 12% sucrose + 1.5 % deoxyglucose (DG) medium at 65 °C to isolate thermophilic and simultaneously derepressed mutants.

The serial dilutions of expressed cells were plated on sucrose-DG-plates to get approximately 30 colonies per plate. The selected colonies (50) were subsequently replica-plated on sucrose+ DG agar plates. The colonies were individually flooded with glucose oxidize reagent and colonies surrounded by a pink halo were picked and screened by measuring diameter of pink halo around each colony. One mutant strain produced substantially higher ethanol at 65 °C and was designated *K. marxianus* M15. The ethanol secretion was tested in the presence of different sugar concentrations (10,12,15,17 %, w/v).

Batch-culture studies

Molasses (100, 150, 170 g l^{-1}), $(NH_4)_2SO_4$ (0.75%), peptone (0.5%) sodium chloride (0.5%) and yeast extract (0.5% , pH = 5.5) in 23-l fermentor at 40 ± 0.2 °C in duplicate, unless mentioned otherwise. Inocula were prepared in 250 ml conical flasks containing 10 g glucose l^{-1} in above sterile medium (pH 5.5) by aseptically transferring cells from single colonies (5 replicates) and grown at 45 °C on an orbital shaker (150 rpm for 24 h). The concentration of the organism was adjusted to contain 2.24 g dry cells l^{-1}.

Fermenter studies were carried out in a micro-processor controlled 23-L stainless steel fermenter (Biostat C5, Braun Biotechnology, Melsungen, Germany with (15-L working-volume vessel) equipped with instruments and controllers for parameters such as agitation, temperature, pH, 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 $(NH_4)_2SO_4$ (75 g l^{-1}). 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 40 h during which foaming was controlled by adding silicone oil as an antifoaming agent. 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, so pH was controlled automatically at 5.5 using KOH.

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 saline. Cell-free supernatant was used for determining ethanol and unfermented sugars. Total sugars in molasses 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, sucrose, acetic acid, succinic 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

Study of kinetic parameters for batch fermentation was calculated as described previously [7]. Empirical approach of the Arrhenius [8] was used to describe the relationship of temperature dependence on growth and product formation.

The value of μ, i.e. specific growth rate was calculated from plot of ln (X) vs time, Various growth kinetics constant were determined by using the following formulae:

Product yield coefficient with respect to cell mass ($Yp/_{x)}$ = dP/dx

Product yield coefficient with respect to substrate (Yp/s) = dP/ds

Specific rate of product formation (q_p) = μ x $Y_{p/x}$

Substrate utilization (q_s) = μ x /$Y_{x/s}$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 as described earlier [9] .

Results and Discussion

Preliminarily, extensive studies were undertaken to optimize ethanol production by varying process conditions like substrate type, pH of the medium, carbon source concentration, oxygen flow rate and nitrogen additives. The traditional (classical) method involved varying one parameter at a time by maintaining pre-optimized fermentation conditions for optimal production of ethanol in 23-L fermentor in duplicate [6].

Fermentation kinetic parameters for growth and substrate utilization are shown in Table-1 and their comparison of organisms wild and mutant of *K. marxianus*, for ethanol production grown in the presence of different temperatures (20-65°C) are shown in Table-1a. For the product formation, kinetic parameters are shown in Table-2 and the comparison results b/w wild and mutant strains are shown in Table-2a.

Glucose, sucrose and molasses were employed to study their effect on growth and production of ethanol by Mutant 15 in time course study shown in Table 3 & 4 and the comparison shown in Table 3a & 4a respectively. Effect of substrate concentration on kinetic parameters revealed that 15 % sugars gave the highest values of all kinetic parameters except $Q_{P,}$ The optimum ethanol (75g/l) was observed after 72h of fermentation with media containing blackstrap molasses (15% total reducing sugars), optimal pH = 5.5 at 40-45 °C. The representative kinetics of product formation by the mutant culture from glucose, sucrose and molasses medium (Fig. 1) indicated that the activity in the case of mutant derivative reached maximum values after 40 h of fermentation. Sucrose and molasses supported only 1.16- to 1.36-fold more synthesis of Ffase than that on glucose. Thus Ffase biosynthesis in the mutant cells and induction level by glucose was comparable with that on sucrose and molasses but glucose repression of enzyme synthesis was completely overcome by mutation. The product yield of Ffase and specific product formation rate (q_p) by mutant is several-fold higher than the reported values by other workers on *Aspergillus spp*, *S. cerevisiae* and their mutants or some recombinants [9-13].

It was observed that maximum values of ethanol (75 g l^{-1}) were obtained with 1.0 vvm aeration rate for 8 h, followed by 0.25 vvm for an other 32 h at 45 °C with 250 rmp agitation speed in 23-L using 5.5 pH fermentors, following growth on 150 g TRS l^{-1} though organism could grow up to 65 °C, while other researchers have done work on yeast of *Kluyveromyces Marxianus* [15,16], which grew only up to 52 °C.

Kinetic parameters showed that the effect of different temperatures and tested organisms on product formation parameters was found to be highly significant. The interactive effect of temperatures and organisms was also found highly significant on Q_p, $Y_{p/s}$ and $Y_{p/x}$. Fig.1 shows the maximum production of ethanol on of different temperatures (40 °C and 45 °C). The effect of sucrose 15%, molasses 12%, molasses 15% and molasses 17%, on ethanol production shown in Fig. 2 and the higher production of ethanol obtained at 15% molasses.

Table 1. **The 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 temperatures under control conditions.**

Temp. ºC	Strain	μ (h^{-1})	Q_x (g cells /l/h)	$Y_{x/s}$ (g cells/g substrate)	Q_s (g/l/h)	q_s (g/g/h)	t_d (h)
20	W	0.10^{fg}	0.780^{abcdef}	0.087^{abc}	1.85^{g}	1.149^{hi}	6.93^{d}
	M	0.20^{de}	0.839^{abc}	0.090^{abc}	2.05^{g}	2.222^{defghi}	3.46^{fg}
25	W	0.12^{f}	0.786^{abcde}	0.085^{abc}	1.86^{g}	1.412^{fghi}	5.78^{e}
	M	0.24^{cd}	0.842^{abc}	0.094^{ab}	2.79^{def}	2.553^{def}	2.89^{gh}
30	W	0.20^{de}	0.810^{abcd}	0.089^{abc}	2.61^{f}	2.247^{defghi}	3.46^{fg}
	M	0.24^{cd}	0.889^{ab}	0.096^{ab}	3.88^{b}	2.500^{defg}	2.89^{gh}
35	W	0.21^{cde}	0.812^{abcd}	0.094^{ab}	3.12^{cde}	2.230^{defghi}	3.30^{fg}
	M	0.26^{bc}	0.890^{ab}	0.100^{ab}	4.11^{b}	2.600^{def}	2.66^{ghi}
40	W	0.23^{cde}	0.814^{abcd}	0.098^{ab}	3.35^{c}	2.347^{defgh}	3.01^{fgh}
	M	0.30^{b}	0.895^{a}	0.102^{ab}	4.76^{a}	2.941^{de}	2.31^{hi}
45	W	0.18^{e}	0.799^{abcdef}	0.099^{ab}	1.33^{h}	2.001^{efghi}	2.48^{hi}
	M	0.36^{a}	0.899^{a}	0.109^{a}	4.99^{a}	3.303^{d}	1.92^{i}
50	W	0.10^{fg}	0.708^{def}	0.078^{abc}	1.26^{hi}	1.282^{ghi}	6.93^{d}
	M	0.30^{b}	0.823^{abcd}	0.100^{ab}	3.98^{b}	3.001^{de}	2.88^{gh}
55	W	0.08^{fgh}	0.67^{4f}	0.075^{abc}	0.83^{ij}	1.066^{i}	8.66^{c}
	M	0.20^{de}	0.764^{bcdef}	0.087^{abc}	2.83^{cd}	2.298^{defghi}	3.46^{fg}
60	W	0.06^{gh}	0.210^{g}	0.005^{de}	0.36^{jk}	12.00^{b}	11.55^{b}
	M	0.19^{e}	0.723^{cdef}	0.054^{bcd}	2.67^{ef}	3.518^{d}	3.65^{fg}
65	W	0.03^{h}	0.133^{g}	0.001^{e}	0.21^{k}	30.00^{a}	23.1^{a}
	M	0.18^{e}	0.675^{ef}	0.039^{cde}	0.34^{k}	4.615^{c}	3.85^{f}
LSD values ($P \leq 0.05$)		0.05218	0.1278	0.521	0.4811	1.261	0.9436

Table1a. **Comparison of organisms (wild and mutant) *Kluyveromyces marxianus* on ethanol production grown at different temperatures.**

***K.marxianus* strains**	μ	Q_x	$Y_{x/s}$	Q_s	q_s	t_d
Wild	0.130^{b}	0.654^{b}	0.071^{b}	1.676^{b}	2.572^{b}	7.407^{a}
Mutant	0.247^{a}	0.824^{a}	0.087^{a}	3.272^{a}	3.926^{a}	3.004^{b}

Table 2. **The kinetic parameters of *K. marxianus* (W) and its mutant strain (M) product formation using ammonium sulphate (0.75%) , pH (5.5) added in molasses medium (15% sugars) at different temperatures under control conditions.**

Temp. °C	Strain	Q_p (g/l/h)	q_p (g/g/h)	$Y_{p/s}$ (g/g subs)	$Y_{p/x}$ (g/g cells)
20	W	1.83^{i}	0.18^{j}	0.16^{g}	1.80^{hij}
	M	3.83^{efg}	0.27^{hi}	0.26^{ef}	1.35^{j}
25	W	2.83^{h}	0.27^{hi}	0.29^{def}	2.25^{gh}
	M	4.18^{cde}	0.31^{ij}	0.38^{b}	1.29^{j}
30	W	3.75^{efg}	0.35^{hi}	0.38^{bc}	1.75^{hij}
	M	4.75^{abc}	0.39^{ghi}	0.48^{a}	1.62^{ij}
35	W	4.05^{ef}	0.45^{fgh}	0.47^{a}	2.14^{hi}
	M	5.18^{ab}	0.59^{ef}	0.51^{a}	2.27^{gh}
40	W	4.90^{ab}	0.72^{de}	0.48^{a}	3.13^{ef}
	M	5.20^{ab}	0.85^{c}	0.51^{a}	2.83^{fg}
45	W	4.13^{cdef}	1.10^{ab}	0.26^{ef}	6.11^{c}
	M	5.28^{a}	1.29^{a}	0.51^{a}	3.58^{de}
50	W	3.50^{fg}	0.81^{cd}	0.13^{g}	7.10^{a}
	M	4.57^{bcd}	1.12^{b}	0.33^{cd}	3.73^{d}
55	W	3.35^{gh}	0.54^{fg}	0.118^{g}	6.75^{a}
	M	4.05^{def}	0.80^{cd}	0.31^{de}	4.00^{d}
60	W	0.13^{j}	0.00^{k}	0.053^{h}	0.00^{k}
	M	3.31^{gh}	0.55^{fg}	0.29^{ef}	2.90^{f}
60	W	0.12^{j}	0.00^{k}	0.012^{h}	0.00^{k}
	M	3.30^{gh}	0.35^{hi}	0.24^{f}	2.00^{hi}
LSD values ($P \leq 0.05$)		0.6412	0.1566	0.052	0.5716

Table 2a. **Comparison of organisms (wild and mutant) of *Kluyveromyces marxianus* on ethanol production grown at different temperatures.**

Organisms	Q_p	$Y_{p/s}$	$Y_{p/x}$	q_p
Parent	2.856^{b}	0.236^{b}	2.202^{b}	0.464^{b}
Mutant	4.364^{a}	0.381^{a}	3.567^{a}	0.677^{a}

Table 3. **The kinetic parameters of *K. marxianus* (W) and its mutant strain(M) for growth and substrate utilization ,using ammonium sulphate (0.75%) , pH(5.5) in the presence of different carbon sources at 40 °C.**

Carbon source (%)		Strain	μ (h^{-1})	Q_x (g cells /l/h)	$Y_{x/s}$ (g cells/g substrate)	Q_s (g/l h)	q_s (g/g/h)	t_d (h)
Glucose	10	W	0.22^{abc}	0.800^{a}	0.085^{a}	4.551^{a}	2.58^{abcd}	3.1^{bcd}
		M	0.23^{abc}	0.825^{a}	0.090^{a}	4.925^{a}	2.55^{abcd}	3.0^{bcd}
	12	W	0.23^{abc}	0.814^{a}	0.088^{a}	4.672^{a}	2.61^{abc}	3.0^{bcd}
		M	0.24^{ab}	0.850^{a}	0.092^{a}	4.954^{a}	2.60^{abc}	2.8^{cd}
	15	W	0.20^{abc}	0.875^{a}	0.095^{a}	4.712^{a}	2.10^{cde}	3.4^{abc}
		M	0.25^{a}	0.895^{a}	0.098^{a}	5.010^{a}	2.55^{abc}	2.7^{d}
	17	W	0.18^{c}	0.840^{a}	0.084^{a}	4.705^{a}	2.14^{de}	3.8^{a}
		M	0.24^{ab}	0.866^{a}	0.094^{a}	4.980^{a}	2.55^{abcd}	2.8^{cd}
Sucrose	10	W	0.20^{abc}	0.755^{a}	0.077^{a}	4.340^{a}	2.59^{abcd}	3.4^{abc}
		M	0.21^{abc}	0.820^{a}	0.082^{a}	4.770^{a}	2.56^{bcde}	3.3^{abcd}
	12	W	0.22^{abc}	0.810^{a}	0.079^{a}	4.530^{b}	2.78^{abc}	3.1^{bcd}
		M	0.23^{abc}	0.831^{a}	0.087^{a}	4.805^{a}	2.64^{abc}	3.0^{bcd}
	15	W	0.23^{abc}	0.825^{a}	0.080^{a}	4.621^{a}	2.87^{a}	3.0^{bcd}
		M	0.24^{ab}	0.850^{a}	0.088^{a}	4.810^{a}	2.72^{a}	2.8^{cd}
	17	W	0.23^{abc}	0.816^{a}	0.082^{a}	4.692^{a}	2.80^{ab}	3.0^{bcd}
		M	0.24^{ab}	0.840^{a}	0.089^{a}	4.820^{a}	2.69^{abc}	2.8^{cd}
Molasses	10	W	0.22^{abc}	0.799^{a}	0.083^{a}	4.550^{a}	2.65^{abc}	3.1^{bcd}
		M	0.23^{abc}	0.825^{a}	0.091^{a}	4.924^{a}	2.52^{abcd}	3.0^{bcd}
	12	W	0.21^{abc}	0.815^{a}	0.084^{a}	4.705^{a}	2.50^{cde}	3.3^{abcd}
		M	0.24^{ab}	0.852^{a}	0.092^{a}	4.970^{a}	2.60^{abc}	3.4^{abc}
	15	W	0.20^{abc}	0.841^{a}	0.085^{a}	4.707^{a}	2.35^{abc}	3.0^{bcd}
		M	0.24^{ab}	0.865^{a}	0.093^{a}	4.970^{a}	2.58^{abcd}	2.8^{cd}
	17	W	0.19^{bc}	0.838^{a}	0.080^{a}	4.710^{a}	2.37^{e}	3.6^{ab}
		M	0.24^{ab}	0.836^{a}	0.090^{a}	4.960^{a}	2.66^{abcd}	2.8^{cd}
LSD values($P \leq 0.05$)			0.0519	0.04830	0.05191	0.9754	0.4153	0.5113

Each value is a mean of two experiments.± stands for between n =2.
Means followed by different subscripts are significantly different at $P \leq 0.05$ using
MStat C software. Carbon source concentrations were 10%, 12%, 15%, and 17% (w/v).
Ammonium sulphate (0.75%) was used as a nitrogen source.

Table 3a. **Comparison of mean values of growth kinetic parameters of wild strain of *K. marxianus* and mutant derivative for substrate utilization parameters in fermentor studies.**

***K.marxianus* strains**	**μ**	**Q_x**	**$Y_{x/s}$**	**Q_s**	**q_s**	**t_d**
Wild	0.208^{b}	0.819^{b}	0.085b	4.499b	2.544b	3.211a
Mutant	0.234^{a}	0.848^{a}	0.091a	4.912a	2.610a	2.917b

Table 4. **The kinetic parameters of *K. marxianus* (W) and its mutant M15 strain (M) for ethanol production using different carbon sources in yeast medium.**

Carbon source (%)		Strain	Q_p (g/l/h)	q_p (g/g/h)	$Y_{p/s}$ (g /g substrate)	$Y_{p/x}$ (g/g cells)
Glucose	10	W	4.52^{bcdef}	1.20^{cde}	0.46^{a}	5.48^{a}
		M	5.10^{abcd}	1.33^{abcde}	0.49^{a}	5.82^{a}
	12	W	4.82^{bcde}	1.26^{abcde}	0.50^{a}	5.50^{a}
		M	5.27^{ab}	1.38^{abcd}	0.51^{a}	5.75^{a}
	15	W	4.84^{bcde}	1.34^{abcde}	0.45^{a}	5.80^{a}
		M	5.79^{a}	1.56^{a}	0.51^{a}	6.25^{a}
	17	W	3.50^{g}	1.39^{abcd}	0.41^{a}	5.60^{a}
		M	4.79^{bcde}	1.53^{ab}	0.51^{a}	6.14^{a}
Sucrose	10	W	4.20^{efg}	1.09^{e}	0.44^{a}	5.45^{a}
		M	4.80^{bcde}	1.15^{de}	0.48^{a}	5.50^{a}
	12	W	4.35^{def}	1.24^{cde}	0.48^{a}	5.65^{a}
		M	4.85^{bcd}	1.29^{abcde}	0.49^{a}	5.65^{a}
	15	W	4.43^{def}	1.31^{abcde}	0.45^{a}	5.72^{a}
		M	4.95^{bcd}	1.38^{abcde}	0.50^{a}	5.75^{a}
	17	W	4.00^{fg}	1.29^{abcde}	0.41^{a}	5.65^{a}
		M	4.40^{def}	1.39^{abcde}	0.50^{a}	5.82^{a}
Molasses	10	W	4.50^{def}	1.21^{cde}	0.47^{a}	5.52^{a}
		M	5.05^{abcd}	1.31^{abcde}	0.48^{a}	5.72^{a}
	12	W	4.70^{bcdef}	1.26^{abcde}	0.45^{a}	5.52^{a}
		M	5.24^{abcd}	1.45^{abcd}	0.51^{a}	6.05^{a}
	15	W	4.00^{bcde}	1.28^{abcde}	0.40^{a}	5.58^{a}
		M	5.20^{abc}	1.47^{abc}	0.49^{a}	6.15^{a}
	17	W	4.50^{cdef}	1.21^{abcde}	0.47^{a}	5.52^{a}
		M	4.65^{bcdef}	1.45^{e}	0.51^{a}	6.05^{a}
LSD values($P \leq 0.05$)			0.7612	0.3200	0.050	0.9066

Each value is a mean of two experiments.± stands for standard error between n =2.
Means followed by different subscripts indicate that they are significantly different at
$P \leq 0.05$ using Stat C software. All three nitrogen sources were used at equimolar carbon concentration basis as 10%,12%,15%,17%.

Table 4a. **Comparison of wild strain of *K. marxianus* and mutant derivative for ethanol production grown on different carbon sources in 23 L fermentor.**

Organisms	Q_p	$Y_{p/s}$	$Y_{p/x}$	q_p
Parent	4.431^{b}	0.456^{b}	5.617^{b}	1.264^{b}
Mutant	5.008^{a}	0.499^{a}	5.783^{a}	1.368^{a}

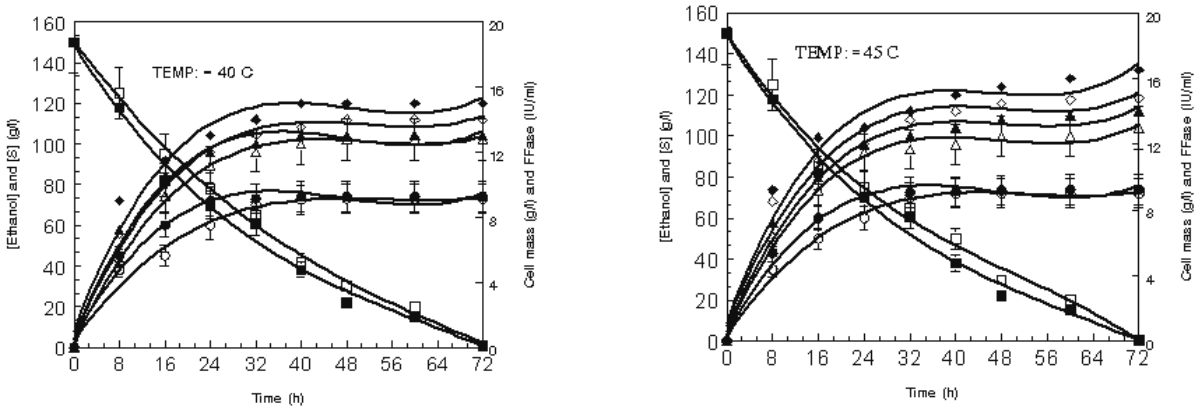

Figure 1. **Effect of different Temperatures (40°C and 45°C) on ethanol formation (○ : W; ●: M), cell mass formed (Δ: W; ▲: M), FFase (◇:W;◆:M)formation and substrate present in fermentor (□:W;■: M) containing 15% sugars in molasses (pH 5.5).**

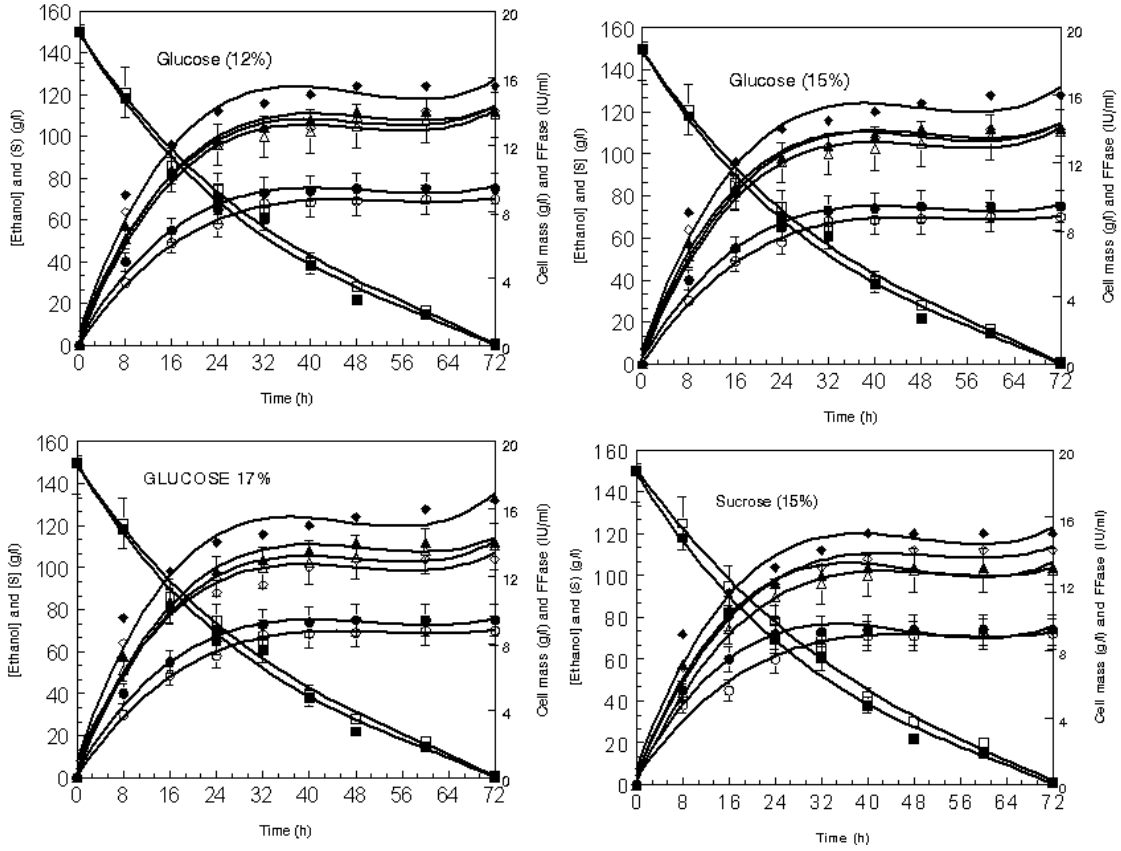

Figure 2. **Effect of different glucose and sucrose concentrations (Glucose 12, 15 & 17%) and sucrose (15%) on ethanol formation (○ : W; ●: M), cell mass formed (Δ: W; ▲: M), FFase (◇:W;◆:M) formation and substrate present in fermentor (□:W;■: M) in batch culture at 40 °C. Error bars show standard error between mean of *n* = 3 observations.**

Conclusion

It is concluded from the study that both wild (W) and mutant (M) strains of *Kluyveromyces marxianus* showed same optimum pH =5.5 but 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.

The large scale production may also be anticipated as economically feasible as the optimized carbon and nitrogen sources are low cost and found in abundance in agricultural countries, like Pakistan. This will also support our industrial scale production proposal.

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