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Enhanced Production of Invertase from Thermoltolerant Yeast Through Black Strap Molasses a Waste Prodcut of Sugar Industry

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Abstract: The current study shows an improved mutant of *S. cerevisiae* selected for the production of Fructofuranosidase (Ffase) from different substrates of industrial waste products in a semi commercial scale digitally controlled bioreactor. Fermentation parameters, such as carbon sources and their concentrations (12-15%), nitrogen sources (0.2%), air flow rate (0.2 vvm), agitational intensity (300 rpm), additives (NaF) were optimized.This gamma ray-induced mutation has given a stable and viable mutant for hyper-production of invertase Ffase.The productivity is >2.0-fold improved over the parental strain on an industrial waste molasses-based medium for extracellular and intracellular Ffase production using semi commercial production conditions. This enhancement occurred due to increase in gene copy number and improvement in gene expression or both or improvement in transport system of the cells. The mechanism underlying this hyper- secretion is of paramount significance and needs further study. The mutant of *S. cerevisiae* has obvious advantage of hyper production of Ffase and may serve as a starting strain for further genetic improvement. It is concluded that this organism may be exploited for bulk production of invertase using molasses an inexpensive and abundant.

Key words:

INTRODUCTION

During the ethanol fermentation process through molasses a conversion takes place through ß-Fructo furanosidase (EC 3.2.1.26) in which sucrose is converted into fructose and glucose. Most of food and pharmaceutical industries utilize this enzyme. The enzyme also possesses fructosyl- transferase activity and can lead to formation of fructo-oligosaccharides which have achieved great attention because of several favourable properties for health foods (Roberfoid 1993; Tomamatau 1994; Yun 1998). A number of cultures make this type of enzyme (Euzenat *et al*. 1997; Muramatsu & Nakakuki 1995; Roberfoid 1993; Yun 1998).

It is very important to screen organisms with the help of sucrose as an inducer for the enhanced production of enzyme at commercial scale. (Hayashi *et al*. 1992).Sucrose is used as sweetener for human consumption and there is no surplus sucrose to be utilized for production of invertase. Its production from molasses could improve economics of ß-fructo-furanosidase (Ffase) production. Sugarcane molasses contains may contain up to 25-40% glucose and fructose which exert catabolic repression on Ffase production (Rincon *et al*. 2001)Enzymatic manufacture of enzymes is prejudiced with the help of insertion and synthesize through catabolite (de Groot *et al.* 2003). Carbon catabolite repression alters with the help of protein, named as CreA (de Vries *et al.* 1999). Sucrose is Ffase inducer and liberates sugars and not feasible for Cre A structure (Hrmova *et al.* 1991).Fungi is also regulated in the same pattern (deGroot *et al.* 2003)

Saccharomyces cerevisiae produces both extracellular and intracellular ß-fructo-furanosidase in submerged fermentation Enhancement in the enzymatic expression of Ffase increases substrate consumption allows for permease (Rincon *et al*. 2001). Isolation of glucoses is regulated through mutants. (Rajoka *et al*. 1998; Haq *et al*. 2001). The separation of this strain is improved and beneficial for the production of ethanol.

Kaiser *et al*. (1986) constructed a series of indicators of the enzyme invertase. Agudo and Zimmermann (1994) observed a low level invertase activity. Vitolo *et al*. (1995) permitted this strain to grow through molasses by variation of parameters like DO,pH and sugar consumption rate.

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Sturm (1996) presented observations for the invertases hydrolyzation from sucrose into glucose and fructose. Zhu *et al.* (1997) created relationship among activity and concentration.Niuris *et al*. (2000) articulated this product and presented its properties.

Tanaka *et al*. (2000) experimental shows that the product is higher in quality form the native cells. Ghosh *et al*. (2001) has purified the invertases and produced high quality of it.Niuris *et al*. (2000) have produced a wide range of microorganisms by utilizing nutrients.Maria *et al*. (2002) purified production of invertases by using a variety of nutrients through SDS-PAGE. Rossi *et al*. (2003) worked on the entrapped cells grown and shown their growth patterns and also found that they are consuming more sugars.Folowing table shows more information regarding its properties:

Table 1: Major raw materials for production of ethanol and invertase

Sugars	Starch	Cellulose and hemi cellulose
Sugarcane	Grains	Wood
Sugar beet	Potatoes	Agricultural residues
Molasses	Root crops	Municipal solid wastes
Fruit		Waste papers, Crop residue

MATERIALS AND METHODS

The research work described in this thesis was conducted in a close collaboration with the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. This research institute is under the administration and management of Pakistan Atomic Energy Commission (PAEC), Islamabad. The substrates, black strap sugar cane molasses was donated by Habib Sugar Mills (Pvt), Nawabshah and Shakarganj Sugar Mills (Pvt), Jhang. Culture was stabilized (through mutagenesis) for higher working temperature and catabolite repression resistant at the same time to make it thermotolerant with retention of hyper-production of ethanol and invertase.

The strain was maintained according to Sirianuntapiboon *et al*. (2004) on yeast growth media in slants and plates, and stored at 4°C (Fig.1). Different chemicals of analytical grade were used for preparation of the yeast minimal growth medium. The chemicals were added into distilled water one by one with shaking and volume was made upto 100 ml in an Erlenmeyer flask of 500 ml capacity. The pH of the media was adjusted to 5.5 by 1 N NaOH and 1 N HCl solutions.

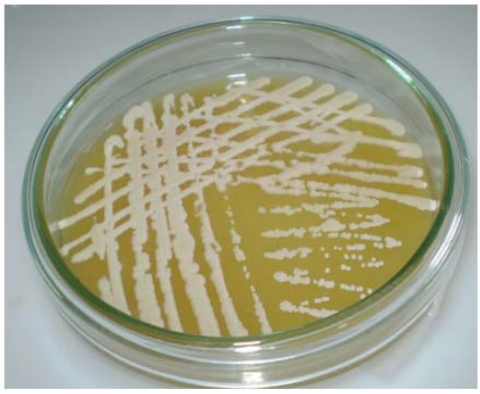

Fig. 1: Fresh Thermotolerant yeast is shown in the Petri plate

The yeast was propagated for 23 liter bioreactors. Then the yeast was propagated for semi-commercial production in two stages to increase the volume. The naming was used as the first stage propagation and the second stage propagation as per Shah (2010).

Harvesting of intracellular invertases:

The intracellular invertases were extracted by sonication from the culture of native and mutant strains of *S. cerevisiae*. After growth of the yeast, the culture was centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was discarded and cell pellet was recovered and washed with distilled water. The cell mass pellet was suspended in normal biological saline (0.89 % w/v of Na Cl). The cells of exact mass were taken for the sonication. They were vortexed to get homogeneous mixing of cells and were disintegrated by ultrasonic waves (10 sec impulses 5 seconds rest for 20 cycles) in ice (To avoid the denaturation of intra-cellar proteins at high

temperature attained during sonication, ice was used during this operation). After sonication, the samples were recentrifuged at 10,000 rpm for about 10 min at 4 °C to settle down the disintegrated cell debris. The supernatant having intracellular invertases was taken and preserved at -20°C.

Invertase assay:

The activity of invertases was determined (by using of 100 µl enzyme 10 m1 MclIVain buffer (0.15 M, pH 5.5) or 50 ml sodium acetate (pH 5.0) and 1.5 (w/v) sucrose solution was used as substrate. The reaction mixture was incubated at 50 °C for 15 min in shaking water bath. Then quenching was performed by placing the reaction mixture in running water for 5 min. The amount of glucose was determined by adding 100 µl of reaction mixture to 1 ml of glucose oxidase based glucose measuring kit (Biocons, Germany) and was incubated at 37 °C for 10 min. Then OD was taken at 500 nm according to the method of Hayashi *et al*. (1992). One unit of invertase activity was equal to µmol of glucose equivalent liberated/ml/min under defined conditions.

RSULTS AND DISCUSSION

Preliminarily, extensive studies were undertaken to optimize Ffase and ethanol production by varying process conditions like substrate type, pH of the medium, carbon source concentration, and nitrogen additives. The traditional classical method involved varying one parameter at a time by maintaining pre-optimized fermentation conditions for optimal production of Ffase and ethanol in 23-l fermenters. Time course studies of invertase production by both wild and mutant organisms from glucose (representative substrate) revealed that after 28 h, the wild organism supported only low level of activity while the mutant organism supported maximum invertase synthesis. Application of Luedeking and Pirt model using specific rate of invertase formation indicated that product formation was both growth and non-growth associated and invertase formation was not a purely primary metabolite. However, a good relationship existed between the enzyme titres and cell mass formation.

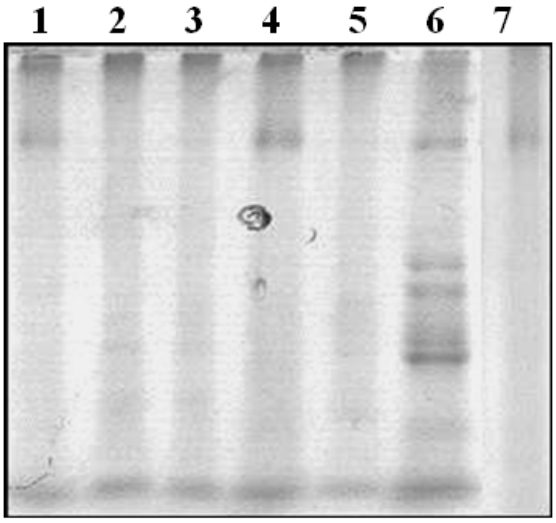

Fig. 2: Protein expression profile with and without sucrose in the growth medium for both wild and derepressed mutant stains of *Saccharomyces cerevisiae*.

Notes: Lane 1 &4= Protein expression profile on 5% glucose of derepressed mutant of *S. cerevisiae;* Lane 2-3 = 5% glucose of wild organism; Lane 5= 5% glucose + 10% sucrose of wild organism; Lane 6= 5% glucose + 10% sucrose of derepressed mutated organism; Lane 7= Invertase standard (from yeast).

As mentioned earlier, the wild and mutant organisms were grown on 5 % glucose, and 5% glucose +10% sucrose media respectively and extra-cellular invertase was checked on 10 % SDS-PAGE (Fig.2). Invertase formation by glucose in the case of wild organism was only marginal as mentioned earlier while the mutant strain showed appreciable amount of invertase (Fig.2, lane 1 and lane 4). When sucrose was also present in the medium, glucose repressed synthesis of invertase in the wild organism but its synthesis in the case of

derepressed mutant was not significantly repressed by glucose. Molasses is being mainly used in our country for production of ethanol. As mentioned earlier, glucose and fructose in molasses cause catabolite repression on invertases (Rincon *et al*. 2001). Different concentrations of sucrose, sucrose and glucose/ fructose combinations and molasses were employed to study their effect on growth and production of extra-cellular and intracellular Ffase from wild and its mutant in time course study. The representative kinetics of Ffase formation by the wild and mutant cultures from 8 % and 10% sucrose (Fig.3 and Fig.4) indicated that the activity in the case of mutant derivative reached maximum values after 24 h of fermentation. At 10% sucrose, the system was saturated with respect to sucrose. The mutant was significantly improved for both intracellular and intracellular Ffase production.

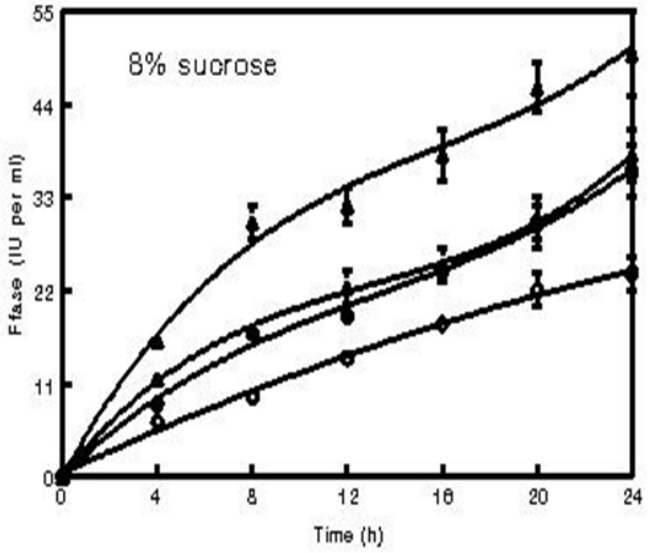

Fig. 3: Extracellular ß-fructo-furanosidase (Ffase) by parental cells (O), and mutant cells (●) and intracellular Ffase by parental (Δ) and mutant cells (▲) following growth on 8% sucrose in 150 l fermenter (working volume 100 l) in yeast growth medium containing molasses substrate. Error bars show standard error between two replicates.

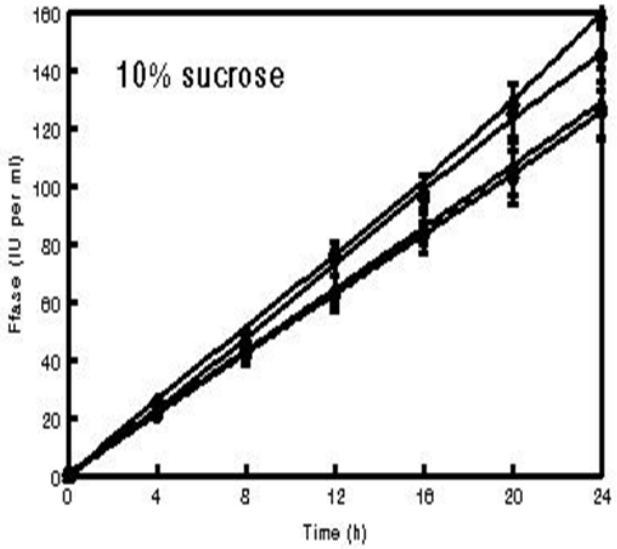

Fig. 4: Extracellular ß-fructo-furanosidase (Ffase) by parental cells (O), and mutant cells (●) and intracellular Ffase by parental (Δ) and mutant cells (▲) following growth on 10% sucrose in 150 l fermenter (working volume 100 l) in yeast growth medium containing molasses substrate. Error bars show standard error between two replicates.

An other representative figure (Fig.4) indicating concomitant production of ethanol, Extracellular and intracellular Ffase production by the mutant revealed that production of both products was growth– and non growth-associated. True time of induction could not be confirmed as the amount of enzyme formed up to 2 h of inoculation in the lag phase was below the accuracy limit of enzyme assays. There were no distinct variation in the values of μ of the parental and mutant (Table 2) cultures on different concentrations of sucrose and but larger variation of Ffase synthesis (Table 2) was noted. This indicated that FFase formation was dependent on μ of both organisms.

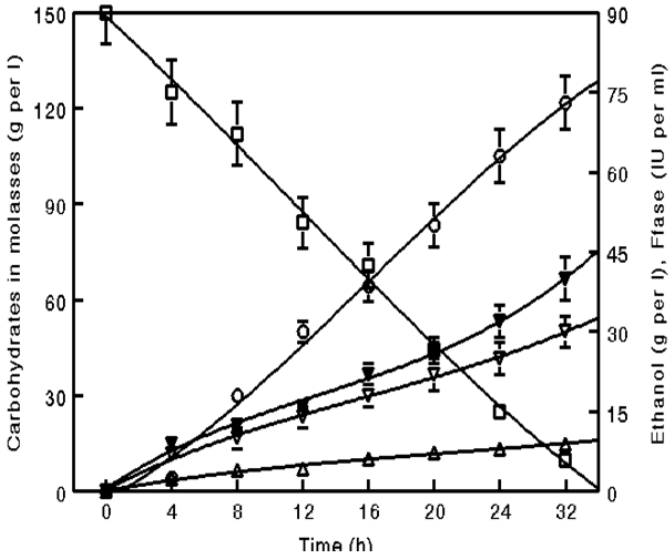

Fig. 5: Kinetics of production of ethanol (O), cell mass (Δ) and molasses in the medium (□), Extracellular (inverted open triangle) and intracellular (▼) following growth of mutant cells on 15% sugars in molasses fermentation medium (pH 5.5)

Table 2: Comparative fermentation kinetic parameters of *S. cerevisiae* and its mutant derivative M9 for extra-cellular and intracellular Ffase production and specific growth rate on different concentrations of sucrose in 150 l fermenter (working volume 100 l) at 40 °C

C. source	Q_p ($Iul^{-1}h^{-1}$)	$Y_{p/S}$ (IU g^{-1})	$Yp_{p/x}$ (IUg^{-1} cell)	μ (h^{-1})
Extracellular Ffase by wild cells				
Suc 8%	650	67	1130	0.21
Suc10%	668	73	1133	0.19
Suc 12%	678	75	1135	0.15
Extracellular FFase by mutant cells				
Suc8%	750	128	1677	0.23
Suc10%	775	132	1556	0.21
Suc 12%	980	136	1690	0.17
Intracellular FFase by wild cells				
Suc 8%	750	261	1911	0.21
Suc 10%	754	265	2377	0.19
Suc 12%	765	268	2378	0.15
Intracellular FFase by mutant cells				
Suc 8%	1050	391	2867	0.23
Suc 10%	1052	400	3566	0.21
Suc 12%	1053	402	3567	0.17

Note: Each value is a mean of n=2 replicates. Standard error between replicates varied between 4-5% of mean values and has not been presented, Suc=sucrose

Table 3: Comparative fermentation parameters of *S. cerevisiae* and its mutant derivative M9 for production of Extracellular and intracellular Ffase production on different substrates in 150 l fermenter at 40 °C

C. source	Q_p ($Iul^{-1}h^{-1}$)	$Y_{p/S}$ (IU g^{-1})	$Yp_{p/x}$ (IUg^{-1} cell)	μ (h^{-1})
Extracellular FFase by wild cells				
Suc.+Glu	510	53	889	0.20
Suc.+Fru	526	53	890	0.20
Molasses	535	54	895	0.20

Table 3: Continue

Extracellular FFase by mutant cells				
Suc.+Glu	1030	101	1677	0.21
Suc.+Fru	1043	98	1556	0.21
Molasses	1045	105	1690	0.21
Intracellular FFase by wild cells				
Sucrose	3458	463	4111	0.20
Suc.+Glu	650	189	1911	0.20
Suc.+Fru	654	143	2377	0.20
Molasses	665	192	1932	0.20
Intracellular FFase by mutant cells				
Suc.+Glu	975	375	3836	0.21
Suc.+Fru	980	400	3900	0.21
Molasses	982	402	3950	0.21

Notes: Each value is a mean of two readings. Standard error between replicates varied between 4-5% of mean values and has not been presented. Glu=glucose and Fru= Fructose.

The phenomena responsible for thermal inactivation of Ffase produced by mutant cells was characterized by an activation enthalpy (H_D^*) of 52.4 kJ/ mol); this is remarkably lower than that for its production by wild cells (70.0 k J/ mol). The value of H_D^* is significantly lower than the values reported for glucose isomerase system (160-235 kJ/mol.

Table 4: Enthalpy and entropy values of extracellular Ffase production and inactivation pathway following growth on molasses at different temperatures.

Organism	ΔH* (kJ/mol)		ΔS*(J/mol.K)	
	Ffase formation	Thermal inactivation	Ffase formation	Thermal inactivation
Parent	67.1	70.0	12.6	-365.4
Mutant	53.5	52.4	-26.4	-428.4

The activation entropy of Ffase formation by mutant cells (-0.0264 k J/mol K) is very low and compares favourably with that of ethanol and xylitol formation reactions needed by a thermotolerant culture (Converti and Dominguez 2001). The activation entropy value of thermal inactivation by mutant cells (-0.428.4 k J/mol K) is also very low and has negative symbol,l which reflects that this inactivation phenomenon implies a little defolding of enzymes of the metabolic network during growth and product formation on molasses at temperature up to 47°C at which the wild culture could not grow. Practically this value is lower than those estimated for alcohol production by thermotolerant systems (Rajoka *et al*. 2005). This suggests a sort of more protection exerted by mutant cell system compared with wild cells against thermal inactivation.

Conclusion:

An improved mutant of *S. cerevisiae* was selected for the production of invertase Fructofuranosidase (Ffase) from different substrates at both laboratory and semi commercial scales through digitally controlled bioreactor of 150 l volume. Optimization of the process was carried out by using a large scale fermentation unit and investigating various fermentation parameters, such as carbon sources and their concentrations (12-15%), nitrogen sources (0.2%), air flow rate(0.2 vvm), agitational intensity(300 rpm), additives(NaF). Gamma ray-induced mutation has given a stable and viable mutant for hyper-production of Ffase (>2.0-fold) improved over the wild type parental strain on an industrial waste molasses-based medium for extracellular and intracellular Ffase production using commercial alcohol production conditions. This enhancement may have occurred either due to increase in gene copy number, or improvement in gene expression or both or improvement in transport system of the cells. The mechanism underlying this hyper- secretion is of paramount significance and needs further study. The mutant of *S. cerevisiae* has obvious advantage of hyper production of Ffase and may serve as a starting strain for further genetic improvement. It is concluded that this organism may be exploited for bulk production of invertase and ethanol using molasses a waste product of sugar industry, which is inexpensive and abundantly available in our country.

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