Alcohol dehydrogenase and invertase activities in ethanol tolerant yeasts

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Two ethanol tolerant yeast isolates of Saccharomyces cerevisiae *Y-10 and* Saccharomyces cerevisiae *Y-7 were compared for their invertase (EC 3.2.1.26, β-fructofuranoside fructohydrolase) and alcohol dehydrogenase (ADH, EC 1.1.1.1, alcohol:NAD oxidoreductase) activities as well as ethanol productivity. The isolates showed significantly higher ethanol productivities compared to the standard strain* Saccharomyces uvarum *and other yeast strains tested. The alcohol dehydrogenase activity was 40–100% higher in the isolates than* S. uvarum *and the percentage change varied depending on the growth conditions. However, this was not true for invertase activity. Both the isolates showed a similar ADH isozyme pattern in contrast to* S. uvarum. *The results suggested that a better correlation between ADH activity and ethanol productivity could be drawn only after extensive studies on the kinetic parameters of the individual isozymes.*

Keywords: Ethanol productivity; molasses fermentation; alcohol dehydrogenase (ADH); isozyme pattern

Introduction

The increasing need of fuel ethanol has prompted extensive investigations for ethanol production from various renewable resources and their efficient utilization by novel microorganisms. Though molasses is a prime source of ethanol, its fermentation efficiency is limited by the sugar and ethanol tolerant capacities of the yeasts. Ethanol inhibition of the fermentation processes is a complex phenomenon and many reports are available on different aspects of this problem.[1-6] Immobilization of yeast cells on solid matrices has been used to obtain higher ethanol productivities by partially overcoming the problem of ethanol inhibition compared to batchwise fermentation.[7,8] Attempts are being made to isolate ethanol and osmo-tolerant yeast strains having better fermentation characteristics from natural sources and by genetic manipulation.[9,10]

With a view to improving the efficiency of the fermentation processes, studies were initiated in our laboratory to isolate substrate and ethanol tolerant yeast strains. Two such isolates of *Saccharomyces cerevisiae* designated as Y-10 and Y-7 were obtained from fermenting sugar-cane juice which showed higher ethanol productivities compared to the standard strain *S. uvarum*.[11] The present paper reports the studies on invertase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase) and alcohol dehydrogenase (EC 1.1.1.1, alcohol:NAD oxidoreductase) levels in these two isolates which play key roles in sugar utilization and ethanol production respectively and their possible role in higher ethanol productivity is discussed.

Materials and methods

Ethanol tolerant yeasts were isolated from fermenting sugar-cane juice and were characterized as *Saccharomyces cerevisiae* at NCYC. They were designated as *S. cerevisiae* Y-10 and *S. cerevisiae* Y-7. *S. uvarum* ATCC 26602 and other yeast strains were obtained from National Collection of Industrial Microorganisms, Poona, India. Sugar-cane molasses obtained from the local sugar factory was analysed for total reducing sugar as described by SivaRaman *et al.*[12] The molasses sample contained 50% (w/v) reducing sugars. The cultures were routinely maintained on a medium containing 20% reducing sugar of cane molasses, yeast extract, 0.3%; malt extract, 0.3% and bacto-peptone, 0.5%. The growth medium (pH 6.5) consisted of either D-glucose, sucrose or reducing sugar of cane molasses at a concentration of 5% in addition to yeast extract, malt extract and bacto-peptone as mentioned above. The fermentation medium (pH 4.5) consisted of either sucrose or reducing sugar of cane molasses at 20- 23% final concentration.

Batch fermentation

Cells grown in 10 ml growth medium containing 5% reducing sugar of molasses for 24 h at 30°C on a rotary shaker were transferred to 90 ml fermentation medium containing 20% molasses sugar. The fermentation was carried out at 30°C under stationary conditions. Samples were withdrawn at 24 and 48 h and ethanol was estimated by ceric ammonium nitrate as well as by gas chromato-

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graphy using Chromosorb 101.[12]

Cells were grown at 30°C on a rotary shaker in growth medium containing the respective sugar. After 20 h cells were harvested by centrifugation and used for the determination of specific ethanol productivity and also for the assay of enzymes.

Specific ethanol productivity

About 1 g (dry weight equivalent) of cells were transferred to fermentation media containing 23% sucrose or reducing sugar of molasses. The flasks were incubated at 30°C and samples were withdrawn at different intervals of time. Ethanol was estimated as mentioned above. Specific ethanol productivity was expressed as ethanol produced per gram dry weight of cells per hour.

Enzyme assays

Yeast cells grown as above (aerobic) or under stationary conditions (anaerobic) were harvested by centrifugation, washed twice in saline and the pellets were frozen in liquid nitrogen. The pellets were slowly thawed and ground well with sand in a precooled mortar. The disrupted cells were suspended in 3 ml buffer (Tris 50 mM, pH 7.5) and the suspension was centrifuged at 12 000*g* for 20 min in a Sorvall RC 5B centrifuge. The supernatant was filtered through glass wool and used for enzyme assays. Invertase activity was assayed according to Gascon and Lampen.[13] The total reaction mixture of 0.5 ml contained 0.2 ml acetate buffer (0.1 M, pH 4.5), 0.1 ml sucrose (0.5 M) and 0.2 ml supernatant. The mixture was incubated at 37°C for 10 min and the reaction was stopped by the addition of 0.5 ml dibasic potassium phosphate (0.2 M) and placed in a boiling water bath for 5 min. Suitable aliquots were used for estimating the D-glucose by the D-glucose oxidase method using Glox reagent. One unit of enzyme activity corresponds to the liberation of one micromole of D-glucose per minute. Alcohol dehydrogenase activity was assayed by the method of Barron and Levine[14] with minor modification. The assay mixture contained Tris–HCl (20 mM, pH 8.6), NAD (1 mM), L-cysteine–HCl (1 mM) in a total volume of 2.8 ml. The reaction was started by the addition of supernatant followed by ethanol (0.6 M). The change in absorbance at 340 nm was monitored every 15 s for one minute. One unit of activity is defined as one micromole of NAD reduced per minute.

Gel electrophoresis

Both the disc and slab gel electrophoresis were carried out in polyacrylamide gels (7%) with the buffer systems of Davis.[15] The gels were stained for ADH activity according to Lutstorf and Megnet[16] except that Tris–HCl (60 mM, pH 8.6) was used instead of pyrophosphate buffer.

Protein was estimated by the method of Lowry *et al.*[17] with bovine serum albumin as standard. NAD, BSA and Tris base were obtained from Sigma Chemical Co., USA. Glox reagent was purchased from Kabi Diagnostica, Sweden. Yeast extract, malt extract and bacto-peptone were from Difco Laboratories, USA. All other chemicals used were of Analar grade.

Results and discussion

Ethanol production by various yeast strains was measured in cane molasses medium and values obtained are given in *Table 1*. Of the strains tested, since all except two showed similar ethanol production at 24 and 48 h these were further screened for their ethanol productivity. The results are given in *Table 2*. The specific ethanol productivities were calculated during the initial phase of fermentation before the ethanol concentration attained inhibitory levels. From the data it is clear that *S. uvarum* and the two isolates *S. cerevisiae* Y-10 and Y-7 show considerably higher ethanol productivities compared to other stains tested. The isolate Y-7 consistently gives higher productivities than *S. cerevisiae* Y-10 both in sucrose and in molasses media. The higher ethanol productivites obtained can be attributed to the greater tolerance towards high sugar and salts present in molasses.

Table 1 Ethanol production by various strains of yeast

Yeast strains	Ethanol %, (w/v) 24 h	48 h
Saccharomyces uvarum	4.48	5.9
Saccharomyces cerevisiae Y-10	4.48	6.3
Saccharomyces cerevisiae Y-7	5.12	7.1
Saccharomyces cerevisiae 3300	6.16	6.8
Saccharomyces cerevisiae 3107	5.14	6.9
Saccharomyces cerevisiae 3176	4.5	5.8
Saccharomyces cerevisiae 3095	2.8	4.1
Schizosaccharomyces pombe 3360	1.19	2.1

Table 2 Specific ethanol productivity during the initial phase of fermentation at 30°C

Yeast strains	Specific ethanol productivity ($g\ g^{-1}\ h^{-1}$) Sucrose	Molasses
Saccharomyces uvarum	0.50	0.44
Saccharomyces cerevisiae Y-10	0.65	0.58
Saccharomyces cerevisiae Y-7	0.88	0.60
Saccharomyces cerevisiae 3300	0.65	0.36
Saccharomyces cerevisiae 3107	0.59	0.31
Saccharomyces cerevisiae 3176	0.58	0.32

The values obtained are the average of three independent determinations

Ethanol affects growth rate, cell viability and in turn the overall fermentation rate which is a complex response that can be considerably influenced both by genetic and environmental factors. The kinetics of ethanol inhibition are further complicated by the exact levels of both intra and extracellular ethanol present in the system.[3,4,6,18] In addition to the effect of ethanol on membrane composition its role on other factors like sugar transport and glycolytic enzymes also has been implicated in ethanol inhibition.[18–20] Though ethanol has been shown to affect several enzymes of the glycolytic pathways[20–22] the inhibition *per se* does not seem to be responsible for the slow fermentation observed. However, the actual levels of the key enzymes present in a cell and their role in the sugar utilization and ethanol production do merit consideration.

We selected the two enzymes namely invertase and alcohol dehydrogenase which play key roles in molasses fermentation and determined their levels in the two isolates

and the standard strain *S. uvarum*. The data in *Table 3* show that the invertase activity is higher in *S. uvarum* and *S. cerevisiae* Y-7 compared to *S. cerevisiae* Y-10. This difference in enzyme activity is observed irrespective of the growth medium used though the specific activity is found to vary with the medium.

The extracellular invertase activity is negligible in all the three strains (data not shown). This clearly indicates that invertase is not one of the rate limiting factors in yeast, unlike in the case of *Zymomonas mobilis* where the enzyme is required for sucrose fermentation more than levan sucrase.[23] The data also suggest that there can be a constitutive and an inducible invertase activity in certain strains of yeast (*Table 3*) but the amount of constitutive enzyme present is far in excess of the level essential for the conversion of substrate needed for ethanol production.

Table 3 Comparison of invertase activity

	Invertase activity (units/mg protein)		
Sugar used	*Saccharomyces uvarum*	*Saccharomyces cerevisiae* Y-10	*Saccharomyces cerevisiae* Y-7
D-Glucose	2.74	0.49	1.64
Sucrose	3.60	0.99	4.22
Molasses	3.28	1.38	4.56

The values obtained are the average of at least three independent experiments

Table 4 Comparison of ADH activity under aerobic and anaerobic conditions

	ADH activity (units/mg protein)		
Sugar used	*Saccharomyces uvarum*	*Saccharomyces cerevisiae* Y-10	*Saccharomyces cerevisiae* Y-7
Aerobic			
D-Glucose	0.319	0.521	0.531
Sucrose	0.388	0.651	0.731
Molasses	0.309	0.493	0.633
Anaerobic			
Sucrose	0.520	0.814	0.897
Molasses	0.427	0.661	0.788

The values obtained are the average of three independent determinations

Table 5 Alcohol dehydrogenase activity in different strains of yeast

	ADH activity (units/mg protein)	
Strain used	Sucrose	Molasses
Saccharomyces cerevisiae 3300	0.345	0.327
Saccharomyces cerevisiae 3107	0.326	0.273
Saccharomyces cerevisiae 3176	0.273	0.312
Saccharomyces cerevisiae 3095	0.319	0.299
Schizosaccharomyces pombe 3360	1.049	0.561

The comparison of specific activities of alcohol dehydrogenase from different strains tested is given in *Table 4*. Both the isolates, *S. cerevisiae* Y-10 and Y-7 show significantly higher alcohol dehydrogenase activity than *S. uvarum* under aerobic and anaerobic conditions. The percentage increase in enzyme activity of the isolates varies from 40–100% depending on the sugar used in growth media. *S. cerevisiae* Y-7 always shows a higher alcohol dehydrogenase activity than the other two strains. A slight inhibition is observed in molasses grown cells often masking the true alcohol dehydrogenase activity which can be attributed to the high salts and other impurities present in molasses.

The data in *Table 5* shows that it is not possible to draw a direct correlation between high alcohol dehydrogenase activity and the increased ethanol productivity exhibited by certain strains of yeasts. For example, *Schizosaccharomyces pombe* shows a very high alcohol dehydrogenase activity compared to other yeast strains but the ethanol production from molasses is found to be low (*Table 1*). This suggests that the nature of the fermentation medium is also a contributing factor for higher ethanol productivity. In other words, *Sch. pombe* has the potential to give higher ethanol productivity based on the high alcohol dehydrogenase activity present provided the proper substrate is used. The same way it can be argued that the lower productivity obtained with different strains of *S. cerevisiae* tested (*Table 3*) can be due to the presence of lower alcohol dehydrogenase activity (*Table 4*). In the studies on vinification by several yeast strains Singh and Kunkee[24] could correlate the level of alcohol dehydrogenase with fusel oil production but not with ethanol. A simple and direct cor-

Figure 1 Gel electrophoresis pattern of ADH isozymes. Tube gels were run and stained for ADH activity as described in Materials and methods. About 50 μg supernatant protein was loaded in each tube. 1, *Saccharomyces cerevisiae* Y-10; 2, *S. uvarum*; 3, mixture of *S. cerevisiae* Y-10 and *S. uvarum*; 4, *S. cerevisiae* Y-7

relation between alcohol dehydrogenase and ethanol productivity seems to be difficult at present due to the complexity of the process and lack of knowledge of other influencing factors.

It is well estabilished that yeast alcohol dehydrogenase has three isozymes namely ADH I (cytosolic), ADH II (oxidative, cytosolic) and ADH III (mitochondrial, with five bands)[25-27] and they are also known to exhibit different kinetic characteristics.[28] To get a better understanding of alcohol dehydrogenase in the isolates the isozyme pattern was studied on polyacrylamide gels. Our results show that *S. cerevisiae* Y-10 and Y-7 have a similar isozyme pattern compared to *S. uvarum* (*Figure 1*). In the electrophoregram only one major band of alcohol dehydrogenase was seen which has lower mobility in both isolates compared to *S. uvarum*. The differences in the minor bands seen in the gels were not very obvious in our studies. Since the kinetic characteristics of the ADH isozymes are known to be different[29] it would be interesting to study the kinetics of the purified isozymes from these isolates. Though the multiplicity of ADH isozymes has been shown by others,[29] the kinetic characterization has not been carried out. The absence of multiplicity of ADH isozyme pattern in *Sch. pombe*[26] in contrast to other *S. cerevisiae* species also suggests that the kinetic parameters like K_m and V_{max} are of more importance.

It is known that the glycerol production is enhanced in partial alcohol dehydrogenase mutants lacking the particular isozyme[30] and the ethanol production is increased in petite mutants lacking mitochondrial isozymes of ADH.[31,32] Hence a more detailed study on the different aspects of ADH and other important enzymes are needed for a better understanding of the complex process of ethanol fermentation.

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