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The Threshold Sugar Level for Yeast Biomass Production from Molasses*

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Abstract

The productivity of yeast biomass in molasses medium is limited by the maximum specific growth rate and the maximum attainable biomass. Normally yeasts growing at high rates in the presence of excessive sugars are affected by catabolite repression, which results in the accumulation of ethanol and a lower yield coefficient. In this communication a relationship between increased productivity and the repressive effect of sugar has been studied during continuous culture of *Candida utilis* on a sugar beet molasses medium. Sugar concentration in the culture medium was increased up to 100 grams per liter and the yeast was cultivated in a chemostat under aerobiosis (air supplemented with oxygen at high biomass concentrations). With the increasing sugar concentration in the medium, the washout point decreased and the ethanol production increased, although the dissolved oxygen level was controlled near 50 per cent of its saturation value. This caused a decrease in yield coefficient and productivity. The specific activities of alcohol dehydrogenase and pyruvate decarboxylase increased as well, indicating that glucose effect may be functional in *C. utilis*, a yeast which has normally been regarded insensitive to glucose effect.

Introduction

The present investigations were undertaken because of the availability of surplus of molasses in Iran. Sugar factories are located widely apart and the production capacity of each factory is not very large. The utilization of molasses at the site of its production is therefore best to avoid transport cost. For this purpose high productivity of yeast biomass becomes invitable in order to make a small scale production unit economically feasible. Moreover, limited availability of agricultural land in Iran makes it imperative to utilize agricultural residues and by-products, such as molasses, as fermentation raw materials.

In a continuous culture system high productivity (grams cells produced per liter of culture per hour) of yeast biomass is limited by the maximum attainable specific growth rate and the maximum biomass concentration of the culture. However, most yeasts are reported to be sensitive to glucose repression in a manner similar to the Crabtree effect.[1] High glucose concentration increases the rate of carbon dissimilation via glycolytic pathway even under aerobic conditions.[2,3] This aerobic fermentation process results in the rapid metabolism of glucose to ethanol and carbon dioxide, and the subsequent high energy produced results in higher specific growth rate[4] in *Saccharomyces cerevisiae*. Although the rate of energy production is high the gain of energy is low. This results in a sugar-carbon

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loss for products other than biomass. These metabolic regulations have been studied on an enzymatic basis by several authors in *S. cerevisiae*.[5-8]

C. utilis on the other hand, is reported to be insensitive to glucose effect[1] with predominantly aerobic metabolic pathways.[9,10] In a previous communication[11] we have reported higher percentage participation of pentose phosphate cycle in the glucose metabolized by *C. utilis* as compared with *S. cerevisiae*. From the metabolic view point *C. utilis* has a better carbon balance towards biomass accumulation as compared with fermentative yeast like *S. cerevisiae*.[12]

This paper reports the production of the biomass of *C. utilis* on Iranian sugar beet molasses which becomes limited due to the changes in metabolic functions of the yeast caused by increasing the sugar concentration in the culture medium.

Materials and Methods

Organism The strain of *C. utilis* CCY 29-38-40 used in these studies was obtained from the culture collection of the Institute of Microbiology, Prague. The yeast was maintained by a monthly subculture on agar slants containing glucose 1%, yeast extract 1%, peptone 1% and agar 2%.

Medium and growth conditions Sugar beet molasses medium of the following composition was used (in grams per liter): sugar (from beet molasses), 20., $(NH_4)_2SO_4$, 10., K_2HPO_4, 7.5., $MgSO_4.7H_2O$, 0.5. In culture media containing different concentrations of sugar, the other components were changed proportionately. For medium preparation the molasses was diluted with tap water and boiled for 10 to 15 minutes with phosphate and sulfuric acid, sufficient to make pH of the solution near 4.5. The molasses solution was filtered and supplemented with rest of the components. The final pH of the medium was 4.5.

Cultivation of the yeast was carried out in a 20 *l* fermenter (Chemap AG., Mannedorf, Switzerland). The fermenter was equipped with a draft tube with impeller at the bottom, which was operated at 1,000 to 2,000 rev/min. Generally 1 to 2 vvm of air was supplied and in some cases the air stream was supplemented with oxygen in order to keep the dissolved oxygen level of the culture near 50 per cent of its saturation value. The dissolved oxygen was monitored with an oxygen electrode. pH of the culture was regulated at 4.5 by the automatic addition of 2N NaOH. Temperature during growth was 30 C. During continuous culture experiments, the dilution rate (D) equals specific growth rate (μ), was controlled by adjusting the flow rate of the medium.

Preparation of cell-free extract Steady-state samples (5 to 10 generations of growth under unchanged environmental conditions) were harvested at 4 C. The cells were washed twice with ice cold 0.06 M phosphate buffer of pH 6.5 and mixed with 10 ml of precooled glass beads of 0.4 to 0.5 mm diameter. The mixture was violently agitated by a vibromix stirrer for 5 min, which resulted in 95 per cent cell disruption. The temperature was kept at 2 to 5 C throughout the operation. The glass beads were sedimented out and the cell debris and the unbroken cells were centrifuged at $2,000 \times g$ for 15 minutes. The cell-free extract was immediately used for the measurement of enzyme activities.

Estimation of enzyme activities Glucose-6-phosphate dehydrogenase was measured according to the method of Kornbrg and Horecker,[13] phosphofructokinase by the method of Racker,[14] glyceraldehyde-3-phosphate dehydrogenase activity was determined according to Warburg and Christian,[15] pyruvate decarboxylase according to Holzer and Goedde,[16] isocitrate dehydrogenase according to Ochoa,[17] and alcohol dehydrogenase according to Racker.[18]

Analytical methods Yeast dry matter was measured by filtering and drying the cells to constant weight. Protein analysis was performed by using the method of Lowry *et al.*[19] Ethanol was measured according to modified method of Winnick,[20] sugar by Schaffer and Somogyi's method,[21] and gaseous exchange measurement was performed by manometric techniques.

Results and Discussion

Figure 1 indicates the pattern of specific activities of some of the enzymes involved in glycolysis, pentose phosphate cycle, aerobic fermentation and tricarboxylic acid cycle. It is clear that in *C. utilis* all the metabolic routes are operative irrespective of the dilution

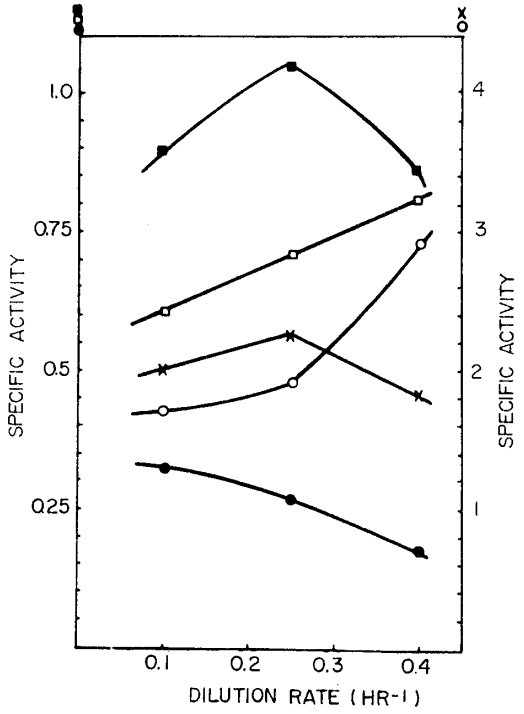

Fig. 1. Enzyme patterns of *C. utilis* grown at different dilution rates under aerobiosis in a culture medium containing 20 g/*l* sugar. The specific activities (micromoles per milligram protein per minute) correspond to the steady-state samples, (■—■) isocitrate dehydrogenase, (□—□) pyruvate decarboxylase, (×—×) glucose-6-phosphate dehydrogenase, (○—○) glyceraldehyde-3-phosphate dehydrogenase, (●—●) phosphofructokinase.

rate studied during continuous culture of the yeast. There is no sudden repression or derepression of the activity of any of the enzymes, rather they show steady and uniform patterns. Under the experimental conditions used for growth of the yeast (2 per cent sugar and chemostatic aerobic growth) aerobic metabolism prevails, even at high dilution rates. Whereas, in other yeasts like *S. cerevisiae* respiratory and tricarboxylic acid cycle enzymes get repressed.[4] These authors reported the onset of the Crabtree effect above dilution rate value of 0.2 hr^{-1}. The value of the glucose consumption rate, which increase with dilution rate, is considered to be a measure of the rate of glycolysis and is informative with respect to the concentration of intermediate metabolites. The repression of the enzyme formation is a function of rate of glucose consumption and subsequent equilibrium rates of metabolic routes.[22] The effect of growth rate on the enzymatic activity has been depicted by other authors.[23]

The high percentage participation of the pentose phosphate cycle in the glucose metabolized by *C. utilis* indicates an obligatory aerobic metabolic route in the yeast.[24] These data, in addition to the gaseous exchange metabolism, show that for the purpose of biomass production from sugary substrates like molasses, *C. utilis* is most suitable, because of its better carbon balance towards biomass accumulation.

Table 1 shows the results of experiments performed to increase the biomass productivity of *C. utilis*. Continuous cultures were run at different dilution rates and at different sugar concentrations in the medium. The maximum productivity of 4.82 g/*l*/hr is achieved in a culture medium containing 50 g/*l* of sugar and at the dilution rate of 0.25 hr^{-1}. Increasing sugar concentration to 100 g/*l* does not result in the increase of the yeast biomass productivity, although the culture has been running under aerobic conditions. Another striking observation is that, when sugar concentration in the culture medium was increased from 20 to 100 g/*l*, the washout point gradually dropped. In other words, decrease in the maximum specific growth rate and a lower actual biomass concentration in the culture which resulted in a decreased productivity. Yield coefficient decreased as well.

Reduced biomass production may be caused by the repression of the aerobic metabolic

Table 1. Relationship between the substrate concentration and the productivity of *C. utilis* during continuous cultivation.

Substrate S_O (g/l)	Dilution rate D (hr^{-1})	Biomass X (g/l)	Productivity DX (g/l/hr)
20	0.1	10.2	1.02
	0.2	10.2	2.04
	0.3	9.8	2.94
	0.4	3.0	1.20
50	0.08	23.8	1.90
	0.15	22.9	3.43
	0.25	19.3	4.82
	0.35	2.3	0.80
100	0.08	39.5	3.16
	0.15	30.0	4.50
	0.20	19.2	3.84
	0.25	7.0	1.75

pathways, which is coupled with the dimnished specific oxygen uptake by the organism.[25] To see whether similar effect is present in the yeast used in the present investigations particularly at high sugar concentrations, experiments were performed with varying sugar contents in the medium in a chemostat and specific oxygen uptake was measured (Table 2). With increasing sugar contents in the medium the specific oxygen uptake near washout decreased sharply, resulting in the repression in oxygen uptake by the yeast. During these experiments the culture was running under aerobic conditions. However, the repression is much lower than in *S. cerevisiae*,[15] where 92 per cent repression was observed at 50 g/l glucose in the medium, while in *C. utilis* 49 per cent repression occurs at a sugar concentration of 100 g/l.

Figure 2 shows that accumulation of ethanol in the culture increases directly with the increase in dilution rate, particularly in media containing a higher percentage of sugar. The high content of ethanol in the culture may have caused earlier washout in cultures with high sugar concentrations.

Table 2. Repression in terms of oxygen uptake near washout (Dc) during continuous cultivation of *C. utilis*.

S_O (g/l)	μl of O_2/hr/mg cells		Repression (%)
	$Q_{O_2}{}^R$	$Q_{O_2}{}^{Dc}$	
10	182.8	175.5	4
20	192.3	186.4	3
30	159.9	145.5	9
50	110.0	82.5	25
100	95.3	48.8	49

S_O=Sugar concentration in the culture medium.
$Q_{O_2}{}^R$=Maximum specific oxygen uptake for respiration (respiratory quotient, RQ=1).
$Q_{O_2}{}^{Dc}$=Specific oxygen uptake near washout or critical dilution rate, Dc.

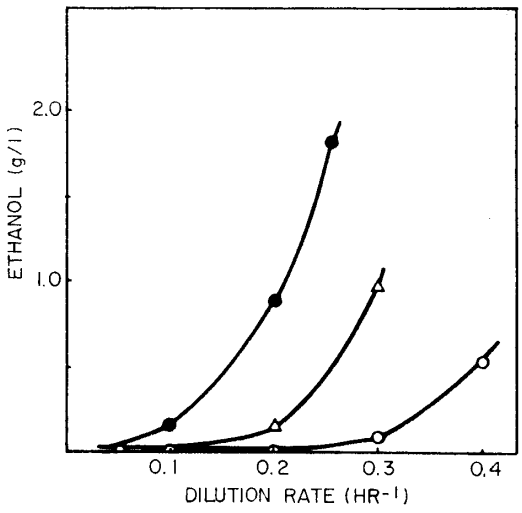

Fig. 2. Effect of dilution rate on the ethanol production by *C. utilis*, grown under aerobic conditions in media containing different concentrations of sugar, (○—○) 20 g/*l*, (△—△) 50 g/*l*, (●—●) 100 g/*l*.

Specific activities of the enzymes leading to ethanol production from pyruvate, i.e., pyruvate decarboxylase and alcohol dehydrogenase were followed in cultures accumulating higher ethanol contents. The results are shown in Figs. 3 and 4. Both the enzymes show an increase in their activities with increasing concentration of sugar in the medium and with dilution rate. However, pyruvate decarboxylase shows a greater increase, which corresponds to earlier results.[26] Three alcohol dehydrogenase isoenzymes have been recognized in *S. cerevisiae*[27] which respond differently to the physiology of the yeast. NAD-dependent alcohol dehydrogenase-I, responsible for ethanol production, increases in its activity when the cells were deriving energy through the fermentative pathway. It is shown in the results presented that the activity of alcohol dehydrogenase corresponds with the ethanol production.

It is concluded that in *C. utilis* there is a threshold level beyond which the increase in sugar concentration in the culture medium results in the fermentative pathway that may be similar to Crabtree effect. Below this level aerobic metabolism predominates irrespective of the dilution rate in a continuous culture system. Productivity of biomass above the

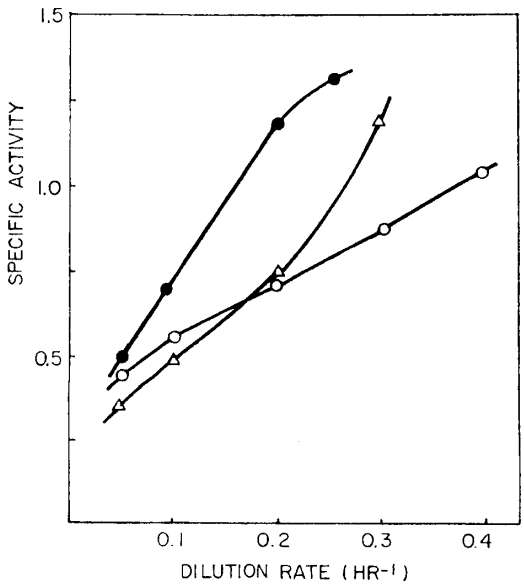

Fig. 3. Patterns of the activities of pyruvate decarboxylase (micromoles per milligram protein per minute) during continuous aerobic growth of *C. utilis*, in response to sugar concentration in the medium, (○—○) 20 g/*l*, (△—△) 50 g/*l*, (●—●) 100 g/*l*.

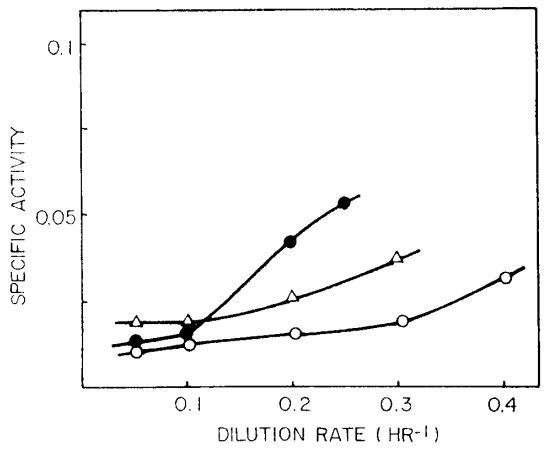

Fig. 4. Patterns of the activities of alcohol dehydrogenase (micromoles per milligram protein per minute) during continuous aerobic growth of *C. utilis*, in response to sugar concentration in the medium, (○—○) 20 g/*l*, (△—△) 50 g/*l*, (●—●) 100 g/*l*.

threshold sugar level declines due to ethanol accumulation which causes earlier washout.

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