Journal of Industrial Microbiology, 8 (1991) 247–252
Elsevier

SIM 00370

The effect of pretreatment on the invertase activity of gel-entrapped yeast biomass

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(Received 17 May 1990; revision received November 1990; accepted 6 July 1991)

Key words: Invertase; Entrapped yeast; Ethanol pretreatment; Heat pretreatment

SUMMARY

Gel-entrapped, non-viable yeast biomass with specific invertase activity has been produced by two different pretreatment protocols: a short-time thermal treatment and a brief contact with concentrated ethanol solutions. Four yeast strains were most promising: *K. fragilis* L-293, *C. utilis* L-282, *S. cerevisiae* L-170 and L-209. Of these, the ethanol-tolerant L-282 and the ethanol-tolerant and heat-resistant L-170 gave the most active gel-entrapped biocatalysts: around 2 mg of reducing sugars produced per mg dry yeast per min.

INTRODUCTION

Yeast invertase is a glycoprotein that catalyses the hydrolysis of sucrose into its monomeric hexoses. In *S. cerevisiae* it exists in several forms which vary not only in molecular mass but also in cell localization; the most abundant is a high molecular mass invertase of about 270 kDa localized outside the cytoplasmic membrane in association with the cell wall [7,12,19].

The conversion of sucrose from sugar cane into glucose and fructose and the further isomerization of glucose into fructose is an interesting alternative to produce high-fructose syrups in tropical countries. The biocatalyst for the hydrolysis reaction in this biological process can be the already commercial available immobilized invertase preparations. However, entrapped whole yeast cells could be a cheaper, more stable and durable biocatalyst [1]. One problem which is encountered with the long-term employment of this biocatalyst is the eventual production of ethanol [9,10]. In order to avoid ethanol production, two alternatives exist: (a) choose a yeast strain that does not produce ethanol, or (b) pretreat the *S. cerevisiae* strains in such a way that the ethanol production capacity is destroyed without impairing the specific invertase activity.

Both approaches have been employed on yeasts having inulinase activity [15,16]. Cell viability has been eliminated by either a short-time thermal treatment or the use of concentrated ethanol solutions. However, similar data have not been published for entrapped whole yeast cells with a high invertase activity. The main purpose of this work was to explore both pretreatment effects upon the specific invertase activity of several yeasts.

MATERIALS AND METHODS

Strains employed

The following yeast strains were used: *Kluyveromyces fragilis* L-293 (DSM 70343), *Candida utilis* L-282 (DSM 70167), *Saccharomyces cerevisiae* L-170 (The Distillers Co. Ltd., Menstrie, U.K., Hybrid b), *S. cerevisiae* L-209 (Instytut Przemyslu Fermentacyjnego, Warsaw, Poland, 1-S.c./39), *Saccharomyces* sp. L-172 (Institut für Mikrobiologie und Biochemie, Geisenheim, F.R.G), *S. cerevisiae* L-181 (CBS 1242), *S. cerevisiae* L-131 (Institut of Chemistry of the Slovak Academy of Sciences, Bratislava, Csechoslovakia, CCY 22-7-1), *Schwanniomyces castelli* L-314 (ibid, CCY 47-3-1), *Rhodotorula glutinis* L-331 (DSM 70398) and *Cryptococcus terreus* L-333 (DSM 70305).

Production of yeast biomass

The yeast strains were maintained in YM agar. For each experiment tubes were incubated at 32 °C for 24 h. The biomass was transferred to 250-ml Erlenmeyer flasks containing 100 ml of a medium of the following composition: glucose, 2%; yeast extract, 0.5%; and peptone, 1%

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[8]. The flasks were left for 24 h at 30 °C and 350 rpm. A subculture was made as above and the incubation conditions repeated. The flasks contents were centrifuged at 3500 rpm at ambient temperature and the separated biomass was washed twice with sterile water.

Biomass pretreatment

The washed yeast biomass was suspended in water to a concentration of 25 mg of dried biomass per ml. It was mixed with an equal amount of an ethanol solution for a specified time. The biomass was then separated by centrifugation at 3500 rpm. Three ethanol solutions were employed: 50, 75 and 95% (v/v); three contacting times were used: 1, 5 and 10 min.

For a thermal treatment the yeast biomass was suspended in water to a concentration of 12.5 mg ml^{-1}. Flasks were immersed in a water bath at 60, 65 and 70 °C for 3, 5 and 7 min, and then rapidly cooled. The biomass was again separated by centrifugation at 3500 rpm.

The concentration of yeast suspensions was determined by measuring its optical density at 600 nm. For each yeast genus and strain a calibration curve was constructed between optical density and biomass dry weight. The latter was obtained by drying at 60 °C until constant weight.

Yeast cell viability was tested by the modified method using Methylene Blue [11].

Gel entrapment

Untreated and pretreated yeast biomass was suspended in water at 12.5 and at 25 mg ml^{-1}. An equal volume of a 3% sodium alginate (Sigma No. A2158 low viscosity) solution was thoroughly mixed and the resulting viscous suspensions were pumped to a manifold with multiple syringes into a 0.1 M $CaCl_2$ solution. Compressed air was also injected into the manifold and went out through the syringes together with the alginate-yeast suspensions. By manipulation of the air flow and the pump setting the diameter of the gelled beads was controlled in all cases around 1 mm. The beads were kept at 7 °C in 0.1 M $CaCl_2$ solution.

Specific invertase activity assay

Beads were thoroughly washed in distilled water and were left for 30 min in 0.2 M, pH 4.6 acetate buffer. 30 ml of sucrose at 20% were placed in a water bath at 50 °C. Separately a known volume of beads was mixed with a known amount of 0.2 M acetate buffer at 50 °C. The two solutions were mixed and left for 15 min. The sum of both volumes was always 30 ml and each value was adjusted in order to have in all cases an amount of yeast biomass entrapped in the beads between 170 and 180 mg of dry weight. One unit of specific activity was equivalent to the amount (mg) of reducing sugars produced per min at the conditions of the assay per mg of dry yeast biomass present. Reducing sugars were analysed by the DNS method [14]. In order to arrive at these proportions a series of preliminary tests were done in which two bead volumes (6 and 12 ml) and yeast suspensions of three concentrations (12.5, 25 and 50 mg ml^{-1} were explored and the yeast *S. cerevisiae* L-170 employed. Initial rate of reaction data were obtained for each case by following the amount of reducing sugars produced every 5 min for 35 min. The rates were then plotted vs. the dry weight of yeast biomass present. The figures for the latter quantity mentioned previously were in the range where reaction rate varied linearly with yeast biomass dry weight. Our results agreed well with recent data by Cuban researchers [4].

Statistical analysis

Analysis of variance and multiple comparison of means by the Tukey HSD test were done employing the SYSTAT package.

RESULTS AND DISCUSSION

Invertase activity of different yeast strains

There was a significant variation in invertase activity for the tested, gel-entrapped yeast strains as can be seen in the data shown in Table 1. The differences of about two orders of magnitude among the groups were consistent for several cell preparations of each strain. Differences were

TABLE 1

Invertase activities of entrapped yeast biomass without any pretreatment

Yeast strain	Activity[a]
K. fragilis L-293	1.82 + 0.16[b]
C. utilis L-282	1.81 + 0.24[b]
S. cerevisiae L-170	1.54 + 0.08
S. cerevisiae L-209	1.06 + 0.06
Saccharomyces sp. L-172	0.31 + 0.03[c]
S. cerevisiae L-181	0.25 + 0.04[c,d]
S. cerevisiae L-131	0.20 + 0.03[d]
S. castelli L-314	0.07 + 0.003[e]
R. glutinis L-331	0.03 + 0.003[e]
C. terreus L-333	0.03 + 0.04[e]

[a] As specific units (see text). Average of five different determinations employing different gel beads of the same batch. Student's confidence interval at $a = 0.05$ and $df = 4$.

[b,c,d,e] Statistically the same.

statistically significant among yeast genera, *Saccharomyces* species and *S. cerevisiae* strains. The highest values were found for *K. fragilis* L-293 and *C. utilis* L-282. These values are within the same order of magnitude of data previously reported, mostly for *S. cerevisiae* strains. For example, D'Souza and Nadkarni [5] reported a value of 0.38 specific units for non-pretreated *S. cerevisiae* ATCC 3177 whole biomass. On the other hand, de Alteriis et al. [2] compared *S. cerevisiae*, *C. utilis* and *K. marxianus*, freeze-dried biomass, at 30 °C, as free cells and entrapped in cross-linked gelatin and their results were: 1.40 and 1.08; 0.29 and 0.20; 0.25 and 0.18 specific units respectively. A slight decrease in specific activity between the free cell preparation and the entrapped biomass has always been reported. For example, Dhulster et al. [3] obtained the following values for previously freeze dried *S. cerevisiae* free cells and for cells entrapped in cross-linked gelatin, sodium alginate and K-carrageenan soaked in potassium chloride gels: 1.41, 1.08, 0.83 and 0.61 respectively. Not only the type of polymer used affected the results but also the actual physical shape of the entrapped biomass-gel preparation. Cantarella et al. [1] measured 1.55 specific units at 30 °C for a previously freeze-dried *S. cerevisiae* strain as free cells; 0.95 for the cells entrapped in films of polyalbumin gel; 0.51 and only 0.03 for the cells entrapped in hydrogels of poly-2-hydroxyethylmethacrylate pellets and membrane respectively. The only significantly higher experimental value reported by Toda and Shoda [17] was 8.29 specific units for *Saccharomyces pastorianus* at 47.5 °C.

Invertase activity of ethanol-pretreated yeast biomass

The specific activities of the gel-entrapped biomass for the four most promising yeast strains are shown in Table 2 as a function of pretreatment time for the various ethanolic solutions. Table 3 presents the effect of pretreatment on cell viability for the ethanol pretreated cells. It is observed from both sets of data that biomass pretreatment suppressed or hindered cell growth without significantly impairing invertase activity. However in the case of *K. fragilis* L-293 the enzymatic activity decreased when contact times increased, for ethanol concentrations below 75%.

Cell viability was different for the four yeast strains tested. In some cases most of the cells were either viable or non viable and in others a mixture was seen. *S. cerevisiae* L-209 was very sensitive to ethanol pretreatment; on the other hand *C. utilis* L-282 and *S. cerevisiae* L-170 were highly tolerant. The specific activity means for these tolerant yeasts were statistically equal and significantly higher than *K. fragilis* L-293 and *S. cerevisiae* L-207. However, no difference was observed between viable and non-viable cells.

Ethanol pretreatment has been tested with the same objective on yeast having inulinase activity [15]. Although both enzymes are β-fructosidases they are different in their catalytic activity: inulinase is a 2.1. β-D-Fructan-fructanohydrolase (EC 3.2.1.7) which hydrolyzes fructose moieties from the non-reducing end of fructants and invertase is a β-D-fructo-furanoside-fructohydrolase (EC 3.2.1.26) which specifically hydrolyses sucrose [18].

TABLE 2

Specific activity of ethanol-pretreated entrapped yeast biomass[a]

Yeast strain	Ethanol concentration % (v/v)								
	50			75			95		
	Time (min)			Time (min)			Time (min)		
	1	5	10	1	5	10	1	5	10
K. fragilis L-293	1.68	1.41	0.94	1.62	1.31	1.36	1.05	NA[b]	1.09
C. utilis L-282	2.15	2.11	1.97	2.17	1.90	2.02	1.53	1.92	1.84
S. cerevisiae L-170	2.12	2.20	2.00	2.15	2.02	1.73	1.81	1.95	2.00
S. cerevisiae L-209	1.01	1.14	1.06	1.07	1.07	0.97	1.25	1.03	1.22

[a] The data represent the mean of three independent samples.
[b] Data not available, samples lost during handling.

TABLE 3

Viability of ethanol-pretreated entrapped yeast biomass[a]

Yeast strain	Ethanol concentration % (v/v)								
	50			75			95		
	Time (min)			Time (min)			Time (min)		
	1	5	10	1	5	10	1	5	10
K. fragilis L-293	m	m	m	m	m	nv	nv	nv	nv
C. utilis L-282	v	v	v	m	nv	nv	m	nv	nv
S. cerevisiae L-170	v	v	v	v	v	m	m	nv	nv
S. cerevisiae L-209	nv	nv	nv	nv	nv	nv	nv	nv	nv

[a] v, viable; nv, non-viable; m, mixture of both.

The production of inulinase by *K. fragilis* has been studied in detail [6,13], however, no inulinase activity has been reported for *C. utilis* or *S. cerevisiae*.

Parekh and Margaritis [15] suspended a thick slurry (50 ml) of *K. marxianus* in 450 ml of 95% ethanol and agitated at 220 rpm at 25 °C until all cells were non-viable. This time period was established by plate counts. The cells retained 75% of the original inulinase activity found in non-pretreated cells. If a comparison is made between the invertase activity of non-pretreated *K. fragilis* L-293 cells (Table 1) with the activity values of non-viable cells from Table 2 and 3 (10 min with 75% ethanol and the three samples contacted with 95% ethanol) the means were statistically different and the retained invertase activity of the pretreated cells was 64% of the non-pretreated biomass. This figure is comparable to the data of Parekh and Margaritis [15] for the same yeast genus but for a different strain.

This decrease in specific activity between the non-pretreated and pretreated cells was not observed by the

TABLE 4

Specific activity of heat-pretreated entrapped yeast biomass[a]

Yeast strain	Temperature °C								
	60			65			70		
	Time (min)			Time (min)			Time (min)		
	3	5	7	3	5	7	3	5	7
K. fragilis L-293	2.05	1.30	1.65	NA[a]	1.89	2.02	1.91	1.81	1.71
C. utilis L-282	2.20	1.72	1.55	1.96	2.06	1.38	2.00	NA[b]	2.19
S. cerevisiae L-170	2.00	2.12	2.07	2.13	1.76	2.10	2.07	1.78	1.37
S. cerevisiae L-209	1.34	1.27	1.28	1.25	1.37	1.40	1.32	1.46	1.47

[a] The data represent the mean of three independent samples.
[b] Data not available, samples lost during handling.

TABLE 5

Viability of heat-pretreated entrapped yeast biomass[a]

Yeast strain	Temperature °C								
	60			65			70		
	Time (min)			Time (min)			Time (min)		
	3	5	7	3	5	7	3	5	7
K. fragilis L-293	m	m	m	m	m	m	m	nv	nv
C. utilis L-282	v	m	m	m	m	m	m	nv	nv
S. cerevisiae L-170	v	v	v	v	v	m	m	m	m
S. cerevisiae L-209	v	v	m	v	v	m	m	nv	nv

[a] v, viable; nv, non-viable; m, mixture of both.

highly ethanol tolerant (L-282 and L-170) or sensitive (L-209) yeasts. It might be more specific to the *Kluyveromyces* genera.

Invertase activity of heat-pretreated yeast biomass

In Tables 4 and 5 invertase activity data are shown for the same four yeast strains as a function of heat pretreatment parameters. Again it can be readily seen that at least for three yeasts non-viable cells could be obtained without impairment of invertase activity. *S. cerevisiae* L-170 was found to be quite heat-resistant. The specific activity decreased with contact times for *K. fragilis* L-293 and *C. utilis* L-282, especially at the lower temperatures. The same trend was observed for *S. cerevisiae* L-170 only at the highest temperature value. However, there was an opposite tendency for *S. cerevisiae* L-209, an increase in specific activity especially above 65 °C. The means for *S. cerevisiae* L-170, *C. utilis* L-282 and *K. fragilis* L-293 were equal and significantly higher from *S. cerevisiae* L-209. There was no difference in specific activity of viable and non-viable cells irrespective of the pretreatment for all yeasts with the exception of course of *S. cerevisiae* L-170 for which the comparison could not be made.

Thonart et al. [16] showed that a heat treatment for 5 min between 60 and 75 °C was sufficient to kill cells of *K. fragilis* ATCC 12424 when inulinase activity was at its highest. Moreover they claimed that such treatment increased cell permeability for the inulin substrate making the hydrolysis process more effective. Again if a comparison is made between the enzymatic activity of non-pretreated cells (Table 1) with the activity values of non-viable cells from Tables 4 and 5, there is no significant difference for *K. fragilis* L-293, but there are significant increases in *S. cerevisiae* L-209 and *C. utilis* L-282, 39 and 16% of the original specific activity respectively. These results do not necessarily contradict those of Thonart et al. [16] as there is a large difference in molecular mass between the two enzyme substrates. For sucrose there seems to be no increase for its cell permeability by a thermal pretreatment in *K. fragilis*. The increases in invertase activity shown by the other two yeasts do not imply necessarily that sucrose uptake has been favored. It might be an effect on enzymatic translocation or conformation within the cell.

CONCLUSION

Gel-entrapped yeast biomass of quite different invertase specific activity was obtained when six yeast genera were compared. A larger effort may lead to more active preparations equal to the strain used by Toda and Shoda [17]. The pretreatment conditions explored produced viable and non-viable biocatalysts that retained their specific invertase activity. The highly ethanol tolerant yeasts *C. utilis* L-282 and *S. cerevisiae* L-170, the latter also being heat-resistant, gave the most active gel-entrapped biocatalysts. On the whole, however, it seemed that the effect of these pretreatments on yeast invertase activity were genus and strain-dependent.

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