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EXTRACTION OF INVERTASE FROM *SACCHAROMYCES CEREVISIAE*: EFFECT OF RHEOLOGICAL PARAMETERS

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ABSTRACT

Baker's yeast cells were suspended in 50 mL of 0.154M $NaHCO_3$ and left under agitation of 200cpm in rotary and reciprocal type-shakers at 45°C for 24h. The yields of invertase obtained through reciprocal and rotary shakers were 33.2% and 46.0%, respectively. The turbulent regimen observed in the reciprocal shaker denatured the invertase more intensely than the laminar regimen occurred in the rotary shaker. A correlation was observed between the scale factors (Reynolds and Froude numbers, symbolized as N_{Re} and N_{Fr}, respectively) and the final invertase activity attained. Thereby, N_{Re} = 564 and N_{Fr} =1.20 led to obtain an extract with high total invertase activity (22U).

KEYWORDS: Invertase, baker's yeast, rheology, *Saccharomyces cerevisiae*.

INTRODUCTION

Invertase (E.C. 3.2.1.26) is located into the cell wall of yeasts, (mainly *S. cerevisiae and S. carlsbergensis*), being sucrose its main substrate. Invertase (identified in 1860) together with α-amylase (identified in 1836) is the eldest known enzyme, both have been produced in industrial scale since the beginning of the twentieth century. The first applications of invertase were in the production of inverted sugar syrup and in the conservation of blackstrap sugarcane molasses during storage in tanks before to be used in ethanol production.[1] However, along the years other uses appeared for invertase such as medicine (antimicrobial and antioxidant agent), in probes for analytical sucrose measurement, in ELISA kit for detecting infection in mammals by strains of genus *Candida* – product commercialized by Rockland Immunochemicals Inc[2]- and in confectionary industry.[3][4]

Invertase, as any other cell wall enzyme, can be removed from the cell wall either by mechanical or chemical methods.[5] In spite of the mechanical breakage – conducted either in liquid medium (ultrasound, dyno or colloid mill, Gaulin-Manton and French presses) or in solid medium (ball mill) - is rapid and efficient, it causes the complete cell disruption and the resulting extract contains invertase mixed with thousands of other proteic substances. As invertase at cell wall is naturally segregated from the overall cell proteins, the chemical methods (osmotic pressure change, treatment with acid, detergents or extraction with acetone/toluene, alkaline solution or biphasic systems) allow to remove the enzyme located at the cell wall without cell disruption.[6][7]

Amongst the chemical methods the most gentle extraction method is the agitation of yeast cell suspended in alkaline medium. The success of the extraction depends on the type of salt and pattern of agitation (laminar or turbulent). Moreover, the pattern of agitation must be associated with rheological parameters in order to allow to implement the scale up of this unit operation. Commonly, the numbers of Reynolds (N_{Re}) and Froude (N_{Fr}) derived from Navier-Stokes equation are used as scale up factors, because both numbers describe the size, velocity and physical properties of the system. If two distinct systems have the same N_{Re} and N_{Fr} then both will be described by the same differential dimensional equations.[5][8]

The aim of this work was the determination of N_{Re} and N_{Fr} associated with the highest extraction yield of invertase from baker's yeast cell wall carried out in reciprocal and gyratory shakers.

MATERIAL AND METHODS

MATERIAL

The strain of *Saccharomyces cerevisiae* employed was isolated from commercial baker's yeast (Fleischmann-Royal, São Paulo, Brazil). All reagents were pro-analysis grade and purchased from traditional trademarks.

METHODS

Yeast Propagation

A loopful of the stock culture (yeast maintained at 4^{o}C in agar nutrient 23.0 g/L; glucose 1.0 g/L) was transferred to test tubes containing 10.0 mL of liquid medium (composition: glucose, 10.0 g/L; peptone, 5.0 g/L; yeast extract, 3.0 g/L) and incubated at 30^{o}C for 48h. Two tubes were then used to inoculate 200mL of molasses supplemented medium (2.4 g/L of

$Na_2HPO_4.12H_2O$, 0.075 g/L of $MgSO_4.7H_2O$ and 5.1 g/L of $(NH_4)_2SO_4$) in a 1-L Erlenmeyer flask, which was then incubated at 30°C for 22h in a NBS Gyratory Shaker (frequency = 200 cycles/min).

Three Erlenmeyer flasks (600mL yeast culture) were used to inoculate a 5-L fermenter (New Brunswick Scientific Co.) containing 2.4L sterilized and supplemented molasses medium. The fermentation was carried out at pH 5.5, agitation of 300 cycles/min and 30°C for 9h. After that, the cells were separated by centrifugation (*5,000xg*/20min), washed twice with distilled water. The cake was dried at 30°C and stored at 4°C.

Autolysis

Dried yeast and 0.154M $NaHCO_3$ solution were introduced in a 500mL-conical flask at proportion 1:10. The suspension was left under agitation (150, 200, 250, 300 or 350 cycles/min) in a gyratory or reciprocal shaker at 45°C for 24h. After that, the content of the flask was centrifuged (*10,000xg*/20min) and the supernatant collected (autolyzate).

Partial Purification of Invertase

Autolyzate and 0.13M sodium picrate were mixed in a 500mL-Becker at volumetric proportion of 1:0.4 and left resting for 3h at 30°C. The mixture was centrifuged (*10,000xg*/30min), being the supernatant collected. To the supernatant was added acetone at proportion 3:1 and the precipitate (invertase) was collected by centrifugation (*10,000xg*/20min) and stored at 4°C.

Measurement of Physical Parameters

Reynolds' number (N_{Re})

The Reynolds' number was calculated by the equation:

$$N_{Re} = (4\pi fr^2 d) \div \eta \qquad \text{(Eq. 1)}$$

Where f = frequency of agitation (cycles/min); r = radius of the flask (cm); d= density of suspension (g/cm^3); η = viscosity (cp).

Froude's number (N_{Fr})

The Froude's number was calculated by the equation:

$$N_{Fr} = (2\pi^2 f^2 r) \div g \qquad \text{(Eq. 2)}$$

Where g = acceleration of gravity.

Density

The density was measured by using a 10mL-picnometer coupled to thermometer.

$$d = m \div V \qquad \text{(Eq. 3)}$$

Where m = mass (g); V = volume (cm^3).

Viscosity

The viscosity was measured through Ostwald's viscometer at 25°C. The draining time was measured by a chronometer. The average time of twenty draining times was taken for each sample. Distilled water was used as reference fluid. The viscosity was calculated by the equation:

$$\eta_1/\eta_2 = (t_1.d_1) \div (t_2.d_2) \qquad \text{(Eq. 4)}$$

Where η_1 = viscosity of the sample (cp); η_2 = viscosity of water (cp); t_1 = draining time to the sample (s); d_1 = density of the sample (g/cm^3); t_2 = draining time to the water (s); d_2 = density of the water (g/cm^3).

Analytical Procedures

Protein measurement

The amount of protein was determined by using the conventional Lowry's method. In a test tube 0.5 mL of sample and 5.0 mL of Lowry's alkaline solution were added. The mixture was left resting for 10 min. After that, 0.5 mL of Folin-Ciocalteau reactive was added and 30 min later the color developed was read at 660nm. The absorbance was converted in protein concentration through a standard curve made with bovine albumin solution (0.4 mg/mL), in which the amount of protein varied between 40µg and 200µg. The minimal square linear equation is represented by:

$$Y = 2.175 \times 10^{-3}x - 2x10^{-3} \quad (r = 0.994) \qquad \text{(Eq. 5)}$$

Where Y = absorbance and x = amount of protein (µg).

Reducing sugars measurement

The amount of reducing sugars was determined by Somogy's method. In a Folin-Wu test tube 0.5 mL of sample, 0.5 mL of distilled water and 1.0 mL of cupric-alkaline solution were introduced. The tube was left in boiling water bath for 10 min. After cooling, 2.0 mL of arseno-molibdenium solution was added. The absorbance of the color developed was read at 540nm. The standard curve was obtained by using glucose solution (0.2 mg/mL), in which

the amount of glucose was changed between 40µg and 200µg. The minimal square linear equation is represented by:

$$Y_1 = 2.04x_1 + 1.1x10^{-2} \quad (r = 0.998) \quad (Eq. 6)$$

Where Y_1 = absorbance; x_1 = amount of glucose (µg).

Invertase activity measurement

In a test tube 1.0 mL of 0.3M sucrose, 1.0 mL of 0.05M acetate buffer (pH 4.6), 0.5 mL of distilled water and 0.5 mL of sample were added. After mixing, the tube was left in water bath at 30°C for 10 min. The reaction was interrupted by adding a mixture 1:1 of 0.17M $ZnSO_4$ and 0.3M NaOH. The precipitate was separated by filtration and the amount of reducing sugars presented in the filtrate was measured as described above.

One invertase unit (U) was defined as the amount of enzyme catalyzing the formation of 1mg of reducing sugars per min at pH 4.6 and 37°C. Specific invertase activity was expressed as U/mg protein.

RESULTS AND DISCUSSION

From Table 1 it is noted that the type of agitation affects the concentration of protein and the invertase activity in the autolyzate. The autolyzate obtained from rotary shaker had 39% lower protein and 64% higher invertase activity than those attained through the reciprocal shaker, being both shakers operated at frequency of 200cycles/min.

Table 1: Extraction and partial purification of invertase from *S. cerevisiae* using reciprocal and rotary shaker at 200cycles/min for obtaining the autolyzate.

Parameters	Autolyzate		Picric supernatant		[e]Acetone cake	
	*Re	**Ro	Re	Ro	Re	Ro
Protein (mg/mL)	10.7	6.48	7.91	4.41	6.99	1.31
[a]Volume (mL)	39.5	43.5	46.0	48.0	10.0	25.0
[b]Invertase activity	0.316	0.528	0.267	0.422	0.415	0.422
[c]Specific invertase activity	29.5	81.5	33.8	96.0	59.4	324
Yield (%)	100	100	88.3	98.4	33.2	46.0
[d]PF	-		1.15	1.18	2.0	4.0

[a]Volume of invertase solution attained in each purification step; [b]Invertase activity expressed as mg RS/mL. min; [c]Specific invertase activity expressed as 10^{-3}.(mg RS/min.mg protein); [d]PF = purification factor; [e]Acetone cake (resulting by addition of acetone in the picric supernatant) solubilized in water; *Re =reciprocal shaker; **Ro = rotary shaker.

Although the shakers frequencies were equal to 200cycles/min, the fluid dynamic established inside the flasks differed according to the type of shaker considered, being turbulent and laminar in reciprocal and rotary shaker, respectively. Thereby, the pattern of flux established inside the flasks could lead to autolyzates having distinct invertase activities. As it can be seen from Table 1 the amount of protein present in the autolyzate from reciprocal shaker (10.7 mg/mL) was higher than that in the autolyzate from rotary shaker (6.48 mg/mL). Taking into account that the specific invertase activity in the autolyzate resulting from rotary shaker was 64% higher than that from reciprocal shaker, then the turbulent regimen causes higher invertase denaturation than the laminar regimen.

Focusing on the purification degree at each step of the partial purification of invertase (first and second step represented, respectively, by picric supernatant and acetone cake), the final purification factors were 2.01 and 4.0 to the autolyzate obtained from reciprocal and rotary shaker, respectively (Table 1).

The Reynolds and Froude numbers related to the obtainment of autolyzate by rotary shaker were calculated from equations 1 and 2. However, to apply these equations the terms related to density (d) and viscosity (η) might be determined experimentally (Table 2), whereas terms such as frequency of agitation (f) and radius of the flask (r) are measured through the shaker dial and paquimeter, respectively.

Table 2: Reynolds and Froude's numbers determined each four hours during the autolysis of yeast cells conducted in rotary shaker (frequency = 200cycles/min).

Time (h)	Density (g/cm^3)	Viscosity (cp)	N_{Re}	N_{Fr}
0	1.022	2.104	582.1	1.20
4	1.023	2.192	559.3	1.18
8	1.022	2.216	552.7	1.23
12	1.017	2.289	532.4	1.13
16	1.019	2.189	557.8	1,24
24	1.015	2.034	598.0	1.21
Average	1.020	2.171	563.7	1.198
Standard deviation	0.002944	0.08162	21.09	0.03970
Coefficient of variation	0.28%	3.8%	3.7%	3.3%

From Table 2 it is observed that both density and viscosity of the fluid inside the flask (yeast cells suspended in 0.154M sodium bicarbonate) did not vary significantly during 24h of autolysis carried out in a rotary shaker at 200cycles/min. As a consequence, N_{Re} and N_{fr} had a

similar behavior. The same was observed for agitation frequency varied between 150cycles/min and 350cycles/min (data not shown).

Table 3 shows that there is a correlation between invertase activity and N_{Re} and N_{Fr}, being the highest invertase activity (0.48 mg RS/mL.min) obtained for N_{Re} and N_{Fr} equal to 564 and 1.20, respectively. Moreover, as the agitation frequency increases the invertase activity in the autolyzate decreases, demonstrating not to be reasonable to subject the cells to frequencies over 350cycles/min for invertase extraction. Besides, the frequency of 400cycles/min corresponds to N_{Re} = 1126, which characterizes a turbulent flux.[8] As already observed, autolysis carried out in a reciprocal shaker, in which the flux inside the flask is turbulent at any frequency of agitation led to attain an autolyzate with invertase activity lower than in rotary shaker.

Table 3: Effect of shaking frequency on the efficiency of invertase extraction using rotary shaker.

Parameters	Frequency of agitation (cycles/min)				
	150	200	250	300	350
Protein (mg/mL)	5.26	5.15	4.92	5.51	4.53
[a]Volume (mL)	45.0	45.0	45.0	45.0	46.0
Invertase activity (mg RS/mL.min)	0.365	0.480	0.354	0.210	0.176
Specific activity (mg RS/min.mg Prot)	0.0694	0.0932	0.0719	0.0381	0.0388
Total invertase activity (mg RS/min)	16.4	21.6	15.9	9.45	8.10
N_{Re}	422	564	704	844	985
N_{Fr}	0.672	1.20	1.87	2.69	3.66

[a]Volume of the autolyzate obtained.

CONCLUSION

The data presented led to conclude that there is a correlation of scale factors (N_{Re} and N_{Fr}) and the invertase present into the autolyzate. Thereby, the highest total invertase activity (21.6 U/min) occurred at N_{Re} and N_{Fr} equal to 564 and 1.20, respectively. Moreover, the denaturation of the invertase was greater under turbulent flow than under laminar flow pattern as it can be deduced from the specific invertase activities attained through reciprocal shaker (29.5 x 10^{-3} mg RS/min.mg protein) and rotary shaker (81.5x10^{-3} mg RS/min.mg protein). The downstream sequence of autolyzate treatment, i.e., precipitation with sodium picrate followed by addition of acetone in the picrate supernatant led to a fourfold purification factor.

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