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INTRACELLULAR INVERTASE AND SUCROSE HYDROLYSIS BY CALCIUM ALGINATE ENTRAPPED MUTANT CELLS OF *SACCHAROMYCES CEREVISIAE* NA-47

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ABSTRACT:

Biocatalyst by entrapping the whole mutant yeast cells of *Saccharomyces cerevisiae* NA-47 into calcium-alginate for the production of inverted syrup (glucose and sucrose) from sucrose as substrate. Out of M_1-M_8, the medium M_2 containing glucose (2.0 %) as carbon source was selected for cell growth and intracellular invertase after the time interval of 48 h. The optimum conditions for sucrose hydrolysis were as sucrose (50 %), alginate beads were repeatedly used of 26 days after every 18 h of incubation time. The beads were also stored at 4°C for 6 months without appreciable loss of the invertase activity.

INTRODUCTION:

The invertase from *S. cerevisiae* (EC 3.2.1.26) is an enzyme with special relevance in food technology and is extensively used in the production of invert syrup from sucrose. Enzyme immobilization technique have gained importance with the expansion of biotechnological applications with attention is focused on stability, recovery and reusability of the immobilization enzymes (Neil *et al.,* 2002). Immobilization means associating the biocatalysts with an insoluble matrix, so that it can retain in proper reactor geometry for its economic reuse under stabilized conditions. Immobilization is a technique confining a catalytically active enzyme or cell within a reactor system and preventing its entry into the mobile phase, which carries the substrate and product. Immobilization of microbial cells can be carried out by adsorption, entrapment, covalent binding, and microencapsulation inside calcium alginate gels, calcium pectate gels or adhesion to wool (Groboillot *et al.,* 1994). These are further developed into a number of combined and less frequently used techniques of immobilization and application of cell biocatalysts for industrial biotransformations (Vojtisek and Jirku, 1983). However, proper selection of immobilization techniques and supporting materials is needed to minimize the disadvantages of immobilization.

An efficient way of producing efficient enzyme catalysts is to immobilize whole microbial cells by entrapment in calcium alginate, because this technique is simple and cheap (Hasal *et al.,* 1992). Moreover, alginate gel is an inert molecule, thus preventing contamination, and it also has no toxic effects. Therefore, it is suitable as an immobilization matrix for bio-molecules and microorganisms (Mattiasson, 1983). Entrapment has been extensively used for the immobilization of cells, but not for enzymes. The major limitation of this technique for the immobilization of enzymes is the possible slow leakage during continuous use in view of the small molecular size compared to the cells (Tampion and Tampion, 1987). The present study investigated upon the evaluation of culture medium, effect of substrate concentration on sucrose hydrolysis by entrapping whole yeast mutant cells of *Saccharomyces cerevisiae* NA-47 repeated use and storage stability of beads.

MATERIALS AND METHODS:

Organism and Culture Maintenance: Chemically treated mutant strain of *Saccharomyces cerevisiae* NA-47 was maintained on YPG agar medium containing

yeast extract containing (g/l): yeast extract 3.0, peptone 5.0, glucose 20.0 and agar 20.0. The strain was sub cultured at 2-month intervals on agar slants and kept at 4°C.

Culture Conditions: The cells of *S. cerevisiae* NA-47 were cultivated at 30°C in 250 ml Erlenmeyer flask (200rpm), containing 50 ml of medium having the following composition (g/l): yeast extract 3.0, peptone 6.0 and glucose 20.0. The pH value adjusted to 5.0 with a 5N HCl before sterilization was carried out for 15 min at 121°C. Flasks were then cooled, inoculated with a loopful of yeast culture and incubated in a rotary shaking incubator at 30°C for 48 h. The agitation rate was kept at 200 rpm. The experiments were run parallel in triplicate. The yeast cells were harvested by centrifugation at 6000 rpm for 15 min. The finally harvested cells further proceed for the determination of intra-cellular activity, dry cell mass as well as immobilized into calcium alginate beads for sucrose hydrolysis.

Culture Media: Different culture media (Table 1) have tested for the production of intracellular invertase by *S.cerevisiae* NA-47.

Extraction of Intracellular Invertase: By taking the washed yeast cells in 5.0 ml of acetate buffer the suspension was poured in a chilled mortar and crushed with the help of a pestle by adding 0.5 g sand. The homogeneous mixture was re-centrifuged and clear supernatant was assayed for intracellular invertase enzyme.

Invertase Activity: Invertase activity was determined after Akgol *et al.* (2001). One invertase unit is defined as the amount of enzyme, which releases 1.0 mg of inverted sugar in 5 min at 35°C and pH 5.0. For invertase activity, 2.5 ml acetate buffer (50 mM, pH 5.5) and 0.1 ml sucrose (300 mM) was added into the individual test tubes. The tubes were pre-incubated at 35°C for 5 min. After the addition of 0.1 ml of appropriately diluted enzyme solution, incubation was continued for another 5 min. The reaction mixture was placed in a boiling water bath for 5 min to stop the reaction and then allowed to cool at room temperature. A blank was also run parallel replacing the enzyme solution with distilled water. To 1.0 ml of each reaction mixture 1.0 ml of DNS was added and placed the tubes in boiling water for 5 min. After cooling to an ambient temperature, volume was raised up to 10.0 ml. Transmittance was measured at 546 nm using spectrophotometer.

Dry Cell Mass: Dry cell mass was determined by centrifugation of fermented broth at 6000 rpm for 15 min in pre-weighed centrifuge tubes. After decanting off the supernatant, cells were washed twice with distilled water and oven dried at 105°C for 2 h (Myers *et al.,* 1997). Final weight was noted to obtain dry cell mass.

Immobilization Procedure: The collected cells were resuspended in 0.05 M acetate buffer, pH 5.0 to give a final concentration of 16mg/ml and were mixed with an equal volume of 4% sodium alginate. This mixture was dropped into 1% $CaCl_2$ solution with constant gentle shaking. The so-formed beads were left for 1 h in this solution, then filtered and washed three times with sterile distilled water before use. The size of beads was estimated as 3 mm in diameter.

Sucrose Hydrolysis Conditions: Batch reactors were run in 250ml Erlenmeyer flask containing 800 beads in 100 ml of 40% sucrose solution. The pH was adjusted of 5.0. The flasks were incubated at 30°C for maximum sucrose hydrolysis. After incubation, the beads were recovered, washed with distilled water and re-used in a new batch with fresh sucrose solution. This operation was repeated until beads retain their integrity.

RESULTS AND DISCUSSION:

Saccharomyces cerevisiae cells that are rich in both intracellular and extracellular invertase enzymes are good models for whole cell immobilization. Selection of support material

is more critical in food technology than other applications of immobilization. Recently, entrapment of yeast cells into alginate beads are used widely as it is inexpensive and physiological inert (Tanaka *et al*., 1984). Different media (M_1-M_8) were evaluated for the production of intracellular invertase by mutant strain of *Saccharomyces cerevisiae* NA-47 as shown in Table 2 in shake flasks when glucose was used in different concentrations (1-2 %) in media (M_1-M_4), the maximum invertase production (209 U/g of dry weight) having maximum dry weight (10.4 g/l) was noticed in medium M_2. All the four media containing glucose as carbon source gave better production of enzyme invertase as compared to the remaining other four media (M_5-M_8) in which sucrose was used as carbon source. It is might due to glucose acts as metabolically rapid carbon source than sucrose, caused rapid cell growth and intracellular invertase. Vainstein and Peberdy (1991) investigated the regulation of invertase using different carbon sources. Hsieh and Silva (1998) studied long-term batch cultures in complex yeast/peptone /glucose medium.

Fig 1 shows the rate (8-64 h) of intracellular invertase production by mutant strain of *Saccharomyces cerevisiae* NA-47 in shake flasks. The production of enzyme was increased with the incubation period from 8 to 48 h. Maximum production (213±3 U/g of dry cell mass) having dry cell mass (10.4±0.1 g/l) was achieved after 48 h of inoculation. Further increase in incubation period beyond 48 h, resulted in the decreased invertase productivity, which became low (153±2 U/g of dry cell mass) at incubation period of 64 h. The optimum time course for maximum invertase activity was found to be 48 h.

Time course of sucrose hydrolysis (2-22 h) for invertase production by calcium alginate immobilized yeast cells of mutant strain of *Saccharomyces cerevisiae* NA-47 was also studied in shake flasks (Fig 2). An increase in sucrose hydrolysis was observed with the incubation period from 2 to 22 h. Maximum sucrose hydrolysis (63.40 %) was achieved 18 h after suspending the beads in sucrose solution. A steady decline in sucrose hydrolysis was observed after 18 h of incubation. So, in the present studies, an incubation period of 18 h was optimized for maximum invertase activity. Table 3 shows the effect of different sucrose concentrations (30-70 %, w/v) on sucrose hydrolysis by immobilized yeast mutant cells of *Saccharomyces cerevisiae* NA-47 into calcium alginate. A lower enzyme production for sucrose hydrolysis (49.50 %) was noted at an initial sucrose concentration of 30 % (w/v). Maximum hydrolysis (68.20 %) was however, achieved at sucrose concentration of 50 % (w/v). With the increase in sucrose concentration beyond 50 % (w/v), the sucrose hydrolysis was reduced becoming very low (53.0 %) when 70 % (w/v) sucrose was used. Thus a sucrose concentration of 50 % (w/v) was optimized for subsequent experiments. Farine *et al*. (2001) reported that sucrose concentration exceeding 0.2M. The kinetic behaviour of invertase was shown to deviate from the basic Michelis-Menten kinetics, since the rate of the enzyme catalyzed hydrolysis decreased gradually. This effect has been successively attributed to the limiting water concentration, to substrate inhibition (Combes and Monsan, 1983).

In general, without entrapment, the cells are not stable during storage and their decrease may be explained as natural cellular inactivation over time induced by starvation. Alginate beads were repeatedly used for 26 days after every 18 h of incubation and did not lost their activity till six months, and stored in 0.05 M acetate buffer (pH 5.5) retained 90 % of their initial activity. This finding was similar to Rossi-Alva and Rocha-Leao (2003) that reported that duration of 6 month retained maximum activity.

Table 1: Composition of Different culture media used.

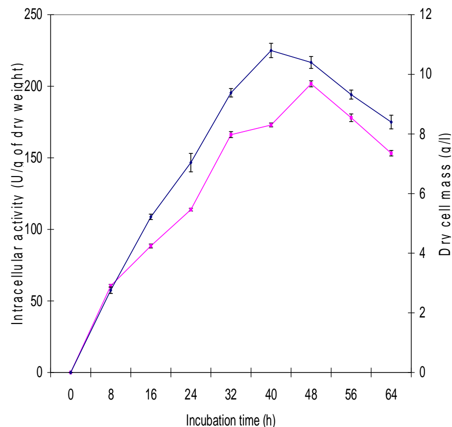
Media ingredients (%)	Culture media used (%, w/v, pH 5.0)							
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8
Yeast extract	0.3	0.3	1.0	1.0	0.3	0.3	1.0	1.0
Peptone	0.6	0.6	1.0	1.0	0.6	0.6	1.0	1.0
Glucose	1.0	2.0	1.0	2.0	-	-	-	-
Sucrose	-	-	-	-	1.0	2.0	1.0	2.0

Table 2: Effect of different media on intracellular invertase production by mutant strain of *Saccharomyces cerevisae* NA-47 in shake flasks.

Media used	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	LSD
Intracellular activity (U/g)	164 ±1	209 ±1	145 ±2	171 ±2	156 ±1	149 ±1	118±2	123±1	0.91
Dry cell mass (g/l)	5.6 ±1	10.4±1	6.1 ±1	10 ±1	5.8±1	6.2 ±1	7.1±1	8.2±1	0.82

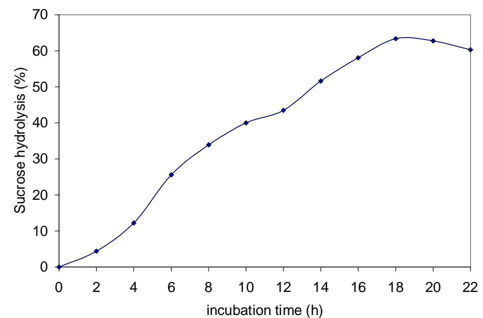
± indicate the standard deviation among the three parallel replicates. The values in each column differ significantly at p≤0.05.

Fig 1:Rate of intracellular invertase production by mutant strain of *Saccharomyces cerevisiae* NA-47



* Temperature 30ºC, glucose concentration 2.0 %, w/v, initial pH 5.0, agitation 200 rpm.
Y-error bars indicate the standard deviation among the three parallel replicates. The values in each set differ significantly at p≤0.05.

Fig 2:Time course study of sucrose hydrolysis by calcium alginate immobilized whole yeast cells of mutant strain of *Saccharomyces cerevisiae* NA-47.



*Temperature 30ºC, sucrose concentration 40 % (w/v), pH 5.0, agitation 200 rpm.

Table 3:Effect of different sucrose concentrations on sucrose hydrolysis by calcium alginate immobilized cells of mutant strain of *Saccharomyces cerevisiae* NA-47*

Sucrose (%)	Sucrose hydrolysis (%)
30	49.5
40	62.9
50	68.2
60	63.9
70	53.0

* Incubation period 18h, temperature 30ºC, pH 5.0, agitation 200 rpm.

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