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Production of extracelluar invertase from *Saccharomyces cerevisiae* strain isolated from grapes

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KEYWORDS

Invertase; Isolation of *S. cerevisiae;* Production Conditions; purification Techniques.

A B S T R A C T

In the present study industrially important yeast *Saccharomyces cerevisiae* was isolated from grape samples using dilution and plating technique. The wild strain was exposed to UV radiation and maintained as improved strain and named as Mutant strain. The wild strain and mutant strains were checked for the invertase production under different conditions, like pH, temperature, different carbon sources, nitrogen sources, phosphate sources. Temperature (20-70) and pH stability (4-9) of enzyme were also studied. The mutant strain should increased production of invertse than the wild type *Saccharomyces cerevisiae* under most of the conditions. Invertase was characterized by SDS – PAGE, Native Gel Electrophoresis and gel filtration chromatogrphy and confirmed as invertase.

Introduction

Enzymes are biocatalyst synthesized by living systems, which are important in synthetic as well as degradative processes. The study of enzymes is an important area, because it exists just on the borderline where the biological and physical sciences meet. Life depends on the complex network of chemical reactions carried out by specific enzymes may have far reaching consequences for the living organism. Most of the industrial enzymes are of microbial origin. The development during the last 25 years has taken place primarily within this group, presumable because the variation in microbial enzymes is wide and because microorganisms can be easily and rapidly

cultivated thus forming an unlimited enzyme source. A wide range of microorganisms produced invertase and can thus utilize sucrose as a nutrient. Commercially, invertase is biosynthesized chiefly by yeast strains of *Saccharomyces cerevisiae* or *Saccharomyces carlsbergensis*.

Invertase is extensively used in confectilonaries, food industries and in pharmaceuticals (Ashokkumar et al., 2001). Microbial invertase is used for the manufacture of calf food and food for honey bees. Many organisms produce invertase such as *Neurospora crassa, Candida utilis, Fusarium oxyspor Phytophthora meganosperma, Aspergillus niger, Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Schwanniomyces occidentalis* (Silveira *et al*., 2000). *Saccharomyces cerevisiae* is the organism of choice for invertase production because of its characteristic high sucrose fermentability. The present study was carried out to produce extracellular invertase enzyme from wild and mutant strains of S.cerevisiae isolated from grape samples.

Materials and Methods

Isolation and identification of invertase producing organism

1g of grape sample was crushed and dissolved in 10 ml of distilled water to make up 10^{-2}. 0.1 ml of the diluted grapes sample was pipetted out and poured in to petriplate containing sucrose agar. Inoculum was spread over the media by using spread plate technique. The plates were incubated at 28°C for 24-48 hours. Identification of the isolated strains was determined according biochemical tests and microscopic studies were carried out to identify the invertase producing efficient yeast.

Development of mutant strain (Strain improvement)

The wild type strain was serially diluted in distilled water up to 10^{-5}. 0.1ml of sample was pipetted out into petriplates containing sucrose agar and spread plated was performed. The plates were exposed to UV source for 30 minutes. The plates were incubated at 28°C for 24-48 hours, and the mutated colonies were taken and examined for enzyme assay.

Measurement of invertase activity

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 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 the tubes placed in boiling water for 5 min. After cooling to 20ºC, volume was raised up to 10 ml. Transmittance was measured at 546 nm using spectrophotometer.

Influence of C,N,P sources on invertase production

The modified sucrose broth was altered with various carbon sources to find out the effect of different carbon sources on invertase production. The carbon sources (were starch, maltose, lactose and Glucose), Nitrogen sources (*The nitrogen*

*sources used for the alteration were urea, sodium nitrate, potassium nitrate, nutrient broth) and Phosphate sources (*Disodium hydrogen phosphate, Diammonium dihydrogen phosphate, Potassium dihydrogen phosphate and sodium dihydrogen phosphate). To find out optimum concentration of all the substrates required for the production of invertase enzymes. The two sets of test tubes were inoculated with wild strain and mutant strain respectively. The corresponding tubes were incubated at 28°C for 24 hours. After the incubation, invertase assay was carried out.

Influence of pH and temperature on invertase production

One set 10ml of sterile modified sucrose broth was adjusted pH 4, 5, 6 and 7 and another set of tubes marked as 20, 30, 40, and 50°C respectively. Inoculum of *S. cerevisiae* were inoculated into test tube. The tubes were incubated at 28°C for 24 hours. After the incubation invertase assay was carried out.

Influence of different concentrations of starch on invertase production

The modified sucrose broth was altered with various carbon sources to find out the effect of different concentrations of starch sources on invertase production(1, 1.5, 2.0, 2.5 and 3.0). To find out optimum concentration starch substrates required for the production of invertase enzymes. The first sets of test tubes were inoculated with wild strain. The second sets of test tubes were inoculated with mutant strain. The corresponding tubes were incubated at 28°C for 24 hours. After the incubation invertase assay was carried out.

Influence of different concentrations of Urea on invertase production

The modified sucrose broth was altered with various concentrations of urea sources from its usual ingredients (0.2, 0.4, 0.6, 0.8 and 1.0) and the altered inoculation medium was prepared. The first sets of test tubes were inoculated wild strain. The second sets of test tubes were inoculated with mutant strain. The corresponding tubes were incubated at 28°C for 24 hours. After the incubation invertase assay was carried out.

Influence of different concentrations of diammonium dihydrogen Phosphate on invertase production

Yeast taken into test tube containing inoculum medium was grown in MSB supplemented with various concentrations of Diammonium dihydrogen phosphate (0.25, 0.5,0.75,1.0 and 1.25) . All the inoculated test tubes were incubated at 28°C for 24hrs. Then enzyme assay was performed after incubation period.

Stability of Invertase enzyme

pH stability of the enzyme

The optimum pH of the enzyme was determined was using different pH buffer (i. e) using acetete (pH 4,5,6,7,8,9), The purified buffers was incubated in these 30 minutes at 28ºC and then assayed for the residual activity for determining its pH stability.Inoculated medium was centrifuged at 5000rpm and supernatant enzyme fluid were only taken. To the enzyme 1ml and 2 ml of buffer were added in a series of test tubes and kept at 28ºC for 30 minutes the invertase producing strain were tested.

Thermal stability of the enzyme

To determine the thermal stability of the invertase enzyme. The medium was centrifuged at 5000rpm and supernatant enzyme fluid was only taken. To the enzyme 1ml and 2ml of Tris acetate buffer was added to make the pH 7. These preparations were taken in a series of test tubes and incubated at different temperature, from 20ºC to 70ºC and the incubation temperature various from 30minutes. This test was done in invertase producing strain and the thermal stability of the enzyme was noted.

Characterization of protein by SDS–Polyacrylamide Gel Electrophoresis [SDS – PAGE]

SDS – PAGE was performed by the method described by Laemmli (1970). From each sample, 25 µl of protein sample was loaded on a polyacrylamide gel. The separating gel (10%) and stacking gel (3.5%) were prepared. Electrophoresis is carried out at a constant voltage of 35 mA through the stacking gel, and at 90 mA through the separation gel at room temperature. After electrophoresis, the gels were stained by Commassive blue. Using standard protein marker, the molecular weight of unknown protein of fungi was determined.

Characterization of invertase enzyme by Native gel electrophoresis

Polyacrylamide gel electrophoresis under native conditions was carried out to evaluate the purity of the extract.12.5 % gel was prepared as described earlier, without the addition of SDS. After the electrophoretic run, proteins were visualized using Coomassie brilliant R-250. Enzyme activity was visualized using α-naphthyl acetate. The enzyme samples were subjected to gel filtration chromatography for purification process.

Results and Discussion

Isolation and screening of invertase producing organisms

The grape samples were collected from Local Market at Thellar and used for isolation of invertase producing yeast. Nearly, about 6 yeast were selected (WS 1-6) based on their enzyme activity and identified as *Saccharomyces cerevisiae* used for the present study. All the isolated organisms were assayed for their invertase activity. The activity varied between 0.0020 IU/ml – 0.1078 IU/ml. Lower level of invertase activity was observed in *Saccharomyces cerevisiae* (WS 1) whereas higher activity was observed in the case of *Saccharomyces cerevisiae* (WS- 2). The high yielding strain WS- 2 was isolated as an efficient strain among the natural isolated. This was well accepted with the previous studies by Ikram –Ul-Haq and Sikandar Ali (2005). In the study, 5 types of yeast strain were kinetically analysed for invertase production. *S. cerevisiae* has great ability to secrete invertase reported by Silveria *et al.* 2000; Shafiq *et al.* (2002).

Although invertase production in filamentous fungi has been studied with the use of the mycelium, on the assumption that it is an intracellular enzyme (Saksena and Bose, 1944; Aritovskaya, 1948; Sainclinier, 1950; De Accadia, Russi, and Bellio, 1955), there is evidence that several filamentous fungi produce extracellular invertase (Gillespie, Jermyn, and Woods, 1952; Crewther and Lennox, 1953; Reese, Birzgalis, and Mandels, 1962; Damle et al., 1958).

Influence of carbon sources on invertase production

Maximum invertase activity was observed in starch containing medium (mutant) with 0.6481 IU/ml than wild type (0.4656 IU/ml).). Maximum amount of invertase was found to be starch for both wild and mutant strains of *Saccharomyces cerevisiae*. These results suggest that the enzyme is a â-D-fructofructofuranoside fructohydrolase, able to attack the â-D-fructofuranosides from the fructose end. Similar behavior of extracellular invertases towards sucrose and raffinose has been detected by many workers (Rubio *et al*., 2002 ; Rashad *et al*., 2006 and Guimaraes *et al*., 2009) using different microorganisms.

Influence of nitrogen sources on invertase production

Table.1 showed higher invertase production in urea containing broth to influence the mutant (0.4856 IU/Ml) than the wild type (0.4656). The result of nitrogen sources were similar to previous research work on nitrogen sources and their concentrations have major effect on enzyme yield because sucrose metabolism shows a specific physiological response to the presence of nitrogen source (Silveira *et al*., 2000). Reduced cell mass might be due to denaturing effect of urea on yeast cells. The reason for high enzyme yield might be positive influence of urease and invertase on each other's secretion into the culture medium.

Effect of Phosphate source on invertase production

In this, maximum invertase production was observed in mutant type with 0.0228 IU/ml than wild type with 0.0118 IU/ml (Table.1). The result of effect of phosphate sources on invertase production were well accepted with earlier studies on the synthesis of β-fructofuranosidase in synchronously dividing cells of *Saccharomyces* continues throughout the budding cycle and follows the increase in cell mass. Similar patterns for cell mass and enzyme increases can be observed even in phosphate-deprived cells which do not divide. Thus, appropriate concentration of phosphate is a critical factor to obtain maximal enzyme secretion.

Effect of phosphate ions on cell mass production and sugar uptake in relation with enzyme secretion was worked out. Product and growth yield coefficients were quite feasible at this point of K_2HPO_4 in the medium. Concentration of readily available phosphate ions in the medium also affects the enzyme productivity and cell mass formation per hour of fermentation period. As concentration of K_2HPO_4 was increased than optimum, cell mass per hour was reduced and same for invertase secretion in the fermentation medium. At high concentrations, phosphate reduces cell mass and increases the alkalinity of medium, which is highly unfavorable for yeast growth and enzyme stability.

Influence of pH on invertase production

The maximum production was observed for wild and mutant strain at pH 7 with 0.0136 IU/ml and 0.0191 IU/ml respectively.(Table.2). An optimum pH was found to be 7.0 for both wild and mutant strains of *Saccharomyces cerevisiae*. This was well accepted with earlier woks by Sachin Talekar *et al.* (2010); Ikram –Ul- Haq and Sikander Ali, (2005); Poonawalla *et al.* (1965); Uma *et al*. (2010).

Table.1 Influence of carbon and nitrogen sources on invertase production

Sources	Invertase activity (IU/ml)	
	Mutant type	**Wild type**
Carbon sources		
Starch	0.6481	0.4656
Maltose	0.0139	0.0071
Lactose	0.0058	0.0024
Glucose	0.6155	0.4656
Nitrogen sources		
Urea	0.4856	0.4656
Sodium nitrate	0.344	0.0332
Potassium nitrate	0.0387	0.0172
Nutrient broth	0.0401	0.0269
Disodium hydrogen phosphate	0.0097	0.0084
Diammonium dihydrogen phosphate	0.0228	0.0118
Potassium dihydrogen phosphate	0.0058	0.0047
Sodium dihydrogen phosphate	0.0226	0.0101

Table.2 Influence of different range of pH and temperature on invertase production

Sources	Invertase activity (IU/ml)	
	Mutant type	**Wild type**
pH range		
4	0.0097	0.0094
5	0.0129	0.0106
6	0.0160	0.0022
7	0.0191	0.0136
Temperature range		
20	0.1862	0.0399
30	0.2345	0.2327
40	0.0425	0.0266
50	0.0582	0.0517

Table.3 Influence of different Concentrations of C, N and P sources on invertase production

Concentrations	Invertase activity (IU/ml)	
	Mutant type	**Wild type**
Starch 1.0	0.3492	0.02328
1.5	1.053	0.1972
2.0	0.8067	0.3957
2.5	3.6460	3.119
3.0	6.1662	4.65
Urea 0.2	0.2328	0.0931
0.4	0.9314	0.7752
0.6	2.6634	1.330
0.8	0.7752	0.1368
1.0	0.2738	0.0580
Na_2HPO_4 0.25	2.3096	1.559
0.5	2.775	1.746
0.75	0.997	0.582
1.0	2.9312	2.328
1.25	0.3580	0.2053

Table.4 pH and thermal stability of the invertase isolated from wild and mutant strains **of** *Saccharomyces cerevisiae*

Stability	Mutant	Wild
pH 4	0.0192	0.0095
5	0.0075	0.0050
6	0.0174	0.0107
7	0.1108	0.0281
8	0.2665	0.1551
9	0.0849	0.0517
Temperature 20	0.0050	0.0042
30	0.0205	0.0147
40	0.0385	0.0342
50	0.0890	0.0600
60	0.2828	0.2506
70	0.0195	0.0175

Table.5 Characterization of yeast protein by SDS –PAGE

S.No	Marker Protein (KDa)	T1 (Mutant)	T2 (Wild)
1	116	130	130
2	97.6	110	102.5
3	66.2	91	59.0
4	42.7	66	37.5
5	31.0	56	27.0
6	20.0	48	-
7	14.4	39.5	-
8	9.5	27.0	-

Figure.1 Characterization of yeast Protein by SDS –PAGE, M- Marker Protein, T1 – Mutant type, T2 – Wild type

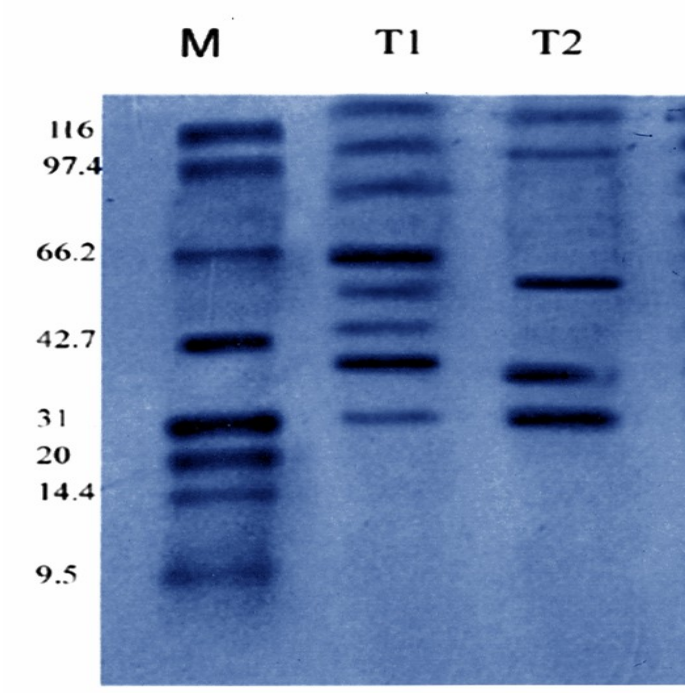
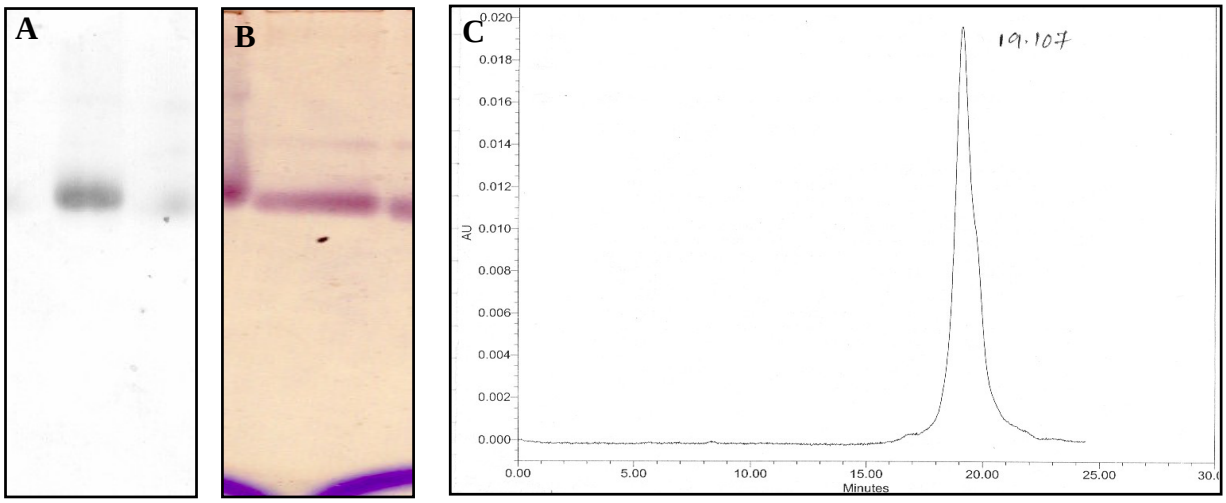


Figure.2 Characterization of Invertase enzyme. A) Invertase enzyme gel (wild); B) Invertase enzyme gel (Mutant) C). Analytical Gel filtration chromatography.



Influence of temperature on invertase production

To observe the influence of temperature of invertase production both mutant and wild strains were maintained at 20, 30, 40 and 50˚C. Low level of invertase was assessed at 40˚C for both wild and mutant. The production was high at temperatures 30˚C for both wild and mutant with 0.2327 IU/ml and 0.2345 IU/ml respectively. Comparatively mutant strain showed increase invertase production in all temperature ranges when compared with wild strain. This result was similar to that reported by Nguyen *et al.* (2005) Rubio *et al*., (2002) ; Guimaraes *et al*., (2007& 2009)and Hussain *et al*., (2009);Rashad *et al*. (2006).

Influence of Starch concentrations on invertase production

To evaluate the influence of different starch sources of invertase production wild and mutant culture were inoculated in indicating reduction in cell mass with an increase in urea concentration, while production media containing 1.0%, 1.5, 2.0, 2.5 and 3.0%. Among the carbon sources more amount of invertase was assayed in soluble starch containing media. Maximum invertase activity was observed in concentrations of 3.0 containing medium (mutant) with 6.1662 IU/ml than wild type (4.656 IU/ml). (Table.3).

Influence of Urea concentrations on invertase production

0.4 concentrations containing media showed higher invertase production in mutant than wild type (0.6) to influence the mutant than the wild type (Table.3). Potombo et al., (1994) reported that the lesser urea concentration is not enough to induce urease in amount sufficient to promote invertase production, and it does not fulfill nitrogen requirement of the yeast thus yielding lesser enzyme. Concentration of urea higher than optimum also produce less amount of invertase, as it induces denaturation of yeast cells this is also supported by Qp and Yx/s , increased enzyme yield at optimal concentration of urea.

Effect of Phosphate concentration on invertase production

In this, maximum invertase production was observed in 1.0 with 2.9312 IU/ml than wild type (2.328 IU/ml) at same concentration of salt.this result were agreed with previous studies by Gines et al.,(2000); Shafiq et al., (2002).

Enzyme characterization.

pH and temperature stability

The enzyme was stable for 30 minutes at pH 8 with 0.2665 (mutant) than 0.1551 in wild type. The results were also closer to the values of Rashad *et al*. (2006) who found that extracellular invertase of *S. cerevisiae* was stable at pH 4.0 – 7.0.Thermal stability of invertase enzyme isolated from *Saccharomyces cerevisiae* was examined at various temperatures, (20, 30, 40, 50 ºC, 60 ºC, 70 ºC) along with various incubation periods (50 minutes). The enzyme was stable at 60 ºC (mutant) with 0.2828 and 0.2506/ml (wild) for nearly 50 minutes (Table.4). The enzyme stability was in the same range of invertases, while it was lower than those from *R. glutinis* (Rubio *et al*., 2002), *A.niger* and *S. cerevisiae* (Rashad *et al*., 2006) which were thermostable up to 60 °C.

Characterization of yeast proteins by SDS-PAGE

Protein profiles of yeast were determined by SDS – PAGE technique. In this study, a maximum of 8 protein bands were observed in *S. cervisiae* (Mutant type) and minimum of 5 bands were observed in *S. cervisiae* (Wildtype). When analyzing the molecular weight, maximum 130 KDa band was observed in both Mutant and wild type of *S. cerevisiae* followed by 110 KDa molecular weight band in Mutant type of *S. cerevisiae* . and 102.5 KDa in wild type (Fig.1.& Table.5). The protein profile of fungi has been already carried out by Burdon and Marshall (1981), Harrington *et al*. (1996), Grigg and Lichtwardt, (1996) and Pointing *et al*., (1999). Fungal phylogenics have been traced on the basis of sequence of protein genes (Loomis and Smith, 1995, Paquin *et al*., 1995). Protein is used to infer phylogeny between organisms (Baladauf and Palmer, 1993; Hasegawa *et al*., 1993; Martin *et al*., 1993; Edlind *et al*., 1996; Zhou and Kleinhof's, 1996).

Characterization of extracellular invertase by Native Gel Electrophoresis

Analysis of Native gel electrophoresis and analytical gel filtration, the purified invertase enzyme were performed. The molecular weight of the purified enzyme estimated by SDS-PAGE indicate that single polypeptide band with Rf value of 7.47 was observed in Mutant type and 7.45 in wild type. When compared with standard, the Mutant type was seemed to be same. In gel filtration chromatography technique, the purified invertase enzyme was found to peak height of 19.107(Fig.2).

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