

## Production, Purification and Characterization of Extracellular Invertase from *Saccharomyces Cerevisiae* NRRL Y-12632 by Solid-State Fermentation of Red Carrot Residue

Mona M. Rashad and Mohamed U. Nooman

Biochemistry Department, National Research Centre, Dokki, 12622, Cairo, Egypt.

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**Abstract:** The production of invertase by cultivating *Saccharomyces cerevisiae* NRRL Y-12632 on some food processing wastes, under solid-state fermentation (SSF) was investigated. The highest productivity of invertase (272.5 U/g dry substrate) was achieved using red carrot (*Daucus carota L.*) jam processing residue inoculated with feeding solution (FS2) designed with seven nutrient components and 90% initial moisture content after 4 days of growth. The enzyme was purified about 29-fold by two chromatographic steps in DEAE- cellulose and Sephacryl S-300. It was stable below 50°C over a pH range (5.0 –7.0) with maximum activity at pH 6.0 and 50°C. The molecular mass of the purified enzyme was found to be 95.5 KDa. The enzyme was able to hydrolyze sucrose and partially raffinose exhibiting  $K_m$  of 60 mM and  $V_{max}$  of 35.5 min<sup>-1</sup> mg<sup>-1</sup> protein. It was completely inhibited by Hg<sup>2+</sup> (1mM) and slightly stimulated by Co<sup>2+</sup> at the same concentration.

**Key words:** Food processing wastes; Red carrot residue; Invertase; Purification; *Saccharomyces cerevisiae*

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### INTRODUCTION

Invertases [ $\beta$ -fructofuranosidases (EC.3.2.1.26)] are members of the GH32 family of glycoside hydrolases, which include more than 370 enzymes of plant and microbial origin (Alberto *et al.*, 2004). They catalyse the hydrolysis of sucrose to produce an equimolar mixture of D-glucose and D-fructose at concentrations lower than 10% sucrose, thus making these enzymes suitable for biotechnological applications (Rubio *et al.*, 2002).

Invertase is extensively used in confectionaries, food industries and in pharmaceuticals (Ashokkumar *et al.*, 2001). It is able to catalyse transfructosylation to produce fructooligosaccharides (FOS) such as kestose (GF2), nystose (GF3) and 1F-fructofuranosyl nystose (GF4). FOS are well known as neo- sugars and has numerous beneficial favourable functional properties (Yun, 1996 and Gill *et al.*, 2006).

The enzymatic activity of invertases has been characterized mainly in plants (Alberto *et al.*, 2004 and Hussain *et al.*, 2009). Several filamentous fungi Invertases have also been characterized such as those from *Aspergillus* sp. (Ashokkumar *et al.*, 2001; Nguyen *et al.*, 2005 and Guimaraes *et al.*, 2007 & 2009). Invertase was also expressed in different yeast strains such as *Candida utilis* (Belcarz *et al.*, 2002), *Pichia anomala* (Rodriguez *et al.*, 1995) and *Rhodotorula glutinis* (Rubio *et al.*, 2002). *Saccharomyces cerevisia* is particularly interesting microorganism, since it synthesizes two Invertases: a glycosilated periplasmic protein and a cytosolic non glycosilated protein (Vitolo *et al.*, 1995 and Rashad *et al.*, 2006).

Increasing concern about pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. The food industry produces large volumes of wastes, both solids and liquids resulting from the production, preparation and consumption of food. Beside their pollution and hazardous aspects, in many cases, food processing wastes might have potential for recycling raw materials or for conversion into useful product of higher value (Sangeetha *et al.*, 2004; Mamma *et al.*, 2008; Rashad and Nooman, 2008 and Guimaraes *et al.*, 2009).

Extensive studies have been done by using synthetic medium for preparation of invertase while a little attention has been paid on its production from unconventional inexpensive sources (Vitolo *et al.*, 1995; Ashokkumar *et al.*, 2001; Rashad *et al.*, 2006 and Guimaraes *et al.*, 2007 & 2009).

Also, the production of enzymes by solid – state fermentation (SSF) have potential advantages over submerged state (SmF) with respect to simplicity in operation, high productivity fermentation, less favourable for growth contaminants and concentrated product formation (Ashokkumar *et al.*, 2001). So the present study

deals with the production, purification and partial characterization of one of the useful industrial enzymes (invertase) by utilization of some Egyptian food processing wastes by *Saccharomyces cerevisiae* NRRL Y-12632.

## MATERIALS AND METHODS

### **Microorganism:**

*Saccharomyces cerevisiae* NRRL Y-12632 was obtained from Agricultural Research Service, Peoria, Illinois, USA. The culture was maintained on a stock slant medium (Wickerman, 1951).

### **Food Processing Wastes:**

Citrus wastes (lemon pulp and orange pulp), sugarcane bagasse, grape juice residue, and apple pomace were obtained from juice processing factories. Red carrot (*Daucus carota L.*) jam processing residue (mixture of roots, leaves and peels) was obtained from Food Technology Research Institute, Vegetables and fruits Processing Centre, Agricultural Research Centre, Giza. Okara (soybean residue, a by-product from the manufacture of soybean milk) was obtained from Food Technology Research Institute, Soy Processing Centre, Agricultural Research Centre, Giza. All wastes were kept at -18 °C until used.

### **Chemical Analysis of Red Carrot (*Daucus Carota L.*) Residue:**

Red carrot residue was analyzed for moisture content, fat, nitrogen content and carbohydrates (A.O.A.C.,1995).

### **Fermentation Media and Growth Conditions:**

Wet food processing wastes were used as the basic substrate for solid state cultivation and fermentation was carried out in 250-ml conical flasks containing 10 g of each substrate and sterilized at 121°C for 20 minutes. The inoculum was prepared by transferring a loopful of *Saccharomyces cerevisiae* from a 7 days old slant to certain volume of stock medium (Wickerman, 1951) or to feeding solution (FS1) containing (g %) sucrose,1 and yeast extract,0.2 (pH 5.5) as described by Sangeetha *et al.*(2004), or to feeding solution (FS2) containing (g %) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4.5; KH<sub>2</sub>PO<sub>4</sub>, 2.3; FeSO<sub>4</sub>, 0.01; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.7; sucrose, 5.0; urea, 1.1; yeast extract, 0.5 (pH 5.0) as described by Ashokkumar *et al.* (2001). The cultures were incubated at 30 °C on a rotary shaker at 200 rpm for 24 h. The substrates were inoculated with 20% (V/W) inoculum and the flasks were incubated in static incubator at 30 °C for 72 h.

### **Preparation of Crude Enzyme:**

At the end of the specified fermentation time, the fermented substrate in each flask was mixed with 50 ml of water and the flasks were agitated in a rotary shaker at 250 rpm for 90 min. The substrate extract was filtered using filter paper (Whatman No.2) and the filtrate was used as crude extracellular enzyme (Sangeetha *et al.*, 2004).

### **Enzyme Assay:**

Invertase activity in the culture filtrate was measured by estimating the liberated reducing sugar released by the hydrolysis of sucrose as described by Somogyi-Nelson method (Somogyi, 1952 and Nelson, 1944) using fructose as a standard. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar (fructose) in 1 min under the assay conditions.

### **Protein Determination:**

Protein was measured according to Lowry *et al.* (1951) method using bovine serum albumin as a standard.

### **Effect of Moisture Content on Invertase Production:**

Invertase production was carried out by growing the organism on the best examined substrates with varying levels of moisture 30, 50,70 and 90% and fermentation was carried out at 30 °C for 72 h as described before.

### **Effect of Incubation Period on Invertase Production:**

Different incubation times (1- 6 days) were used under optimal fermentation conditions at 30 °C. The activity of extracellular enzyme was daily estimated.

***Purification of Invertase:***

***Fractionation by Acetone and Ammonium Sulfate:***

Fractional precipitation with acetone or ammonium sulfate for crude invertase revealed their unsuitability as precipitating agents due to the poor yield obtained relative to the crude enzyme (data not shown).

***Ion-exchange on DEAE- Cellulose:***

The crude culture filtrate obtained by solid-state fermentation was concentrated by lyophilization, and the concentrated enzyme was applied directly on the top of the column (22.5x2.5cm) of preswollen DEAE-cellulose equilibrated with 0.01 M phosphate buffer, pH 7.0. Elution was carried out using the same buffer at a flow rate of 30 ml /h, with a linear gradient of NaCl (0.1-0.5M). Fractions of 10ml were collected and the active enzyme fractions were pooled and dialyzed against the elution buffer for 48 h at 4°C.

***Gel Filtration on Sephacryl S-300:***

The concentrated dialyzed fractions were loaded to a Sephacryl S-300 column (55x1.2cm) equilibrated with 0.1 M phosphate buffer pH 7.0. The fractions of 3 ml were collected at a flow rate of 15ml/h.

***Characterization of the Purified Enzyme:***

***Effect of pH on Activity and Stability of the Pure Enzyme:***

Small aliquots of the purified enzyme were assayed with four buffering agents, namely citrate (pH 3.0-4.0), acetate (pH 4.5-5.5), phosphate (pH 6.0-7.5) and tris-HCl (pH 8.0-9.0) at 0.05 M for each one, for recording pH profile under the standard assay conditions.

To study the effect of pH on the stability, the purified enzyme was preincubated for 30 min. at 30 °C with the four previous buffering systems before testing the enzymatic activities at the standard assay conditions, then relative activity was calculated.

***Effect of Temperature on Activity and Stability of the Pure Enzyme:***

The maximum activity of the pure enzyme was determined at different incubation temperatures ranged from 20-70°C. Thermal stability was studied by incubating the purified enzyme at various temperatures (20-70°C) for 1 h and the remaining enzyme activity was then assayed using the standard assay condition.

***Determination of Substrate Specificity:***

The activity of the purified invertase on several substrates (sucrose, raffinose, trehalose, melezitose, maltose, sorbitol, inulin and glycerol) was tested. All substrates were used in concentration of 0.1 M and determined with reducing sugar assay with fructose as a reference.

***Determination of Michaelis Constant ( $K_m$ ):***

The  $K_m$  value of the purified enzyme was determined according to the method of Hanes-Woolf plot (Hanes, 1932), using sucrose as a substrate.

***Influence of Metal Ions on Enzyme Activity:***

The chemicals which were used namely,  $MgCl_2$ ,  $HgCl_2$ ,  $CoCl_2$ ,  $CuSO_4$ ,  $FeSO_4$ ,  $CaCl_2$ ,  $ZnSO_4$ ,  $BaCl_2$ ,  $NiCl_2$ , preincubated with the purified enzyme at 1mM for 30 min. at 30 °C before determination of the enzyme activities. Blank was taken before adding the metals.

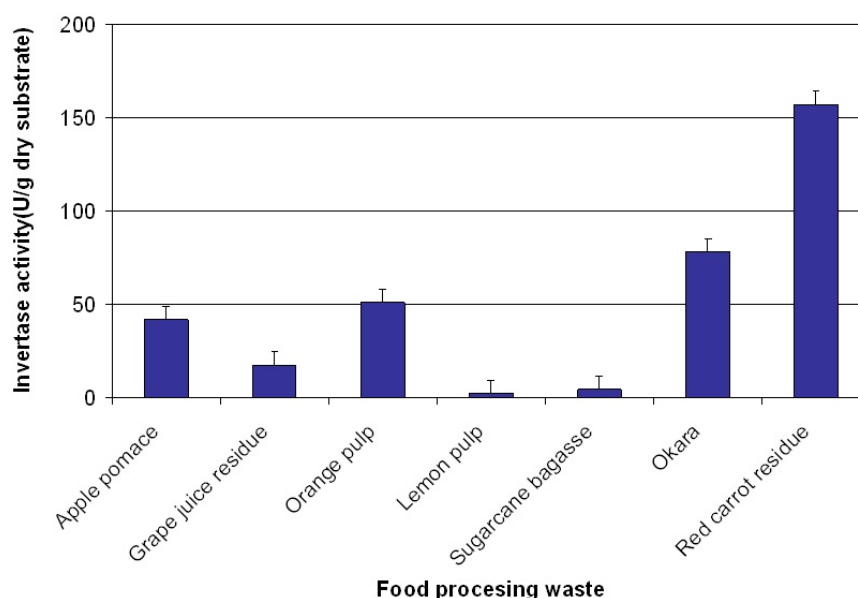
***Molecular Weight Determination Using Gel Filtration Chromatography:***

The molecular weight was determined by gel filtration technique using Sephacryl S-300 (Andrews, 1964). A glass column (55x1.2 cm) loaded with Sephacryl S-300 equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was calibrated with trypsin (23,000), egg albumin (45,000), bovine serum albumin (69,000) and amyloglucosidase (97,000) as standard proteins.

## **RESULTS AND DISCUSSION**

In recent years, the bioconversion of food processing residues matters represents possible and utilizable resources for conversion to useful products. Food processing wastes are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production of bulk chemicals and enzymes using SSF technique (Couto and Sanroman, 2006).

The cultivation of the yeast strain *Saccharomyces cerevisiae* NRRL Y-12632 on seven Egyptian food processing wastes without any additive nutrients using SSF was investigated for the production of extracellular invertase after 3 days of growth (Fig. 1). The data showed a wide variation in the yield of extracellular enzyme through the tested food processing wastes (2.2 – 157.0 U/ g dry substrate). The highest yield of invertase was produced by using red carrot residue as a fermentation medium (157.0 U/g dry substrate), followed by okara, orange pulp, and apple pomace which showed considerable amounts of invertase (78.2, 51.0, and 41.8 U/g dry substrate respectively). Lower yield of extracellular invertase was produced by using grape juice residue, while negligible amounts of the enzyme were detected by using sugarcane bagasse and lemon pulp as a fermentation medium. So, the present study will continue using red carrot residue for production of higher yield of invertase from *S. cerevisiae* by SSF technique. This value (157.0 U/g dry substrate) was certainly higher than that obtained by Mamma *et al.* (2008), who found that the highest invertase activity produced by *A. niger* cultivated on dry orange peel was 72.5 U/g dry substrate at initial pH 5 and 90% moisture content, while the highest activity which was produced by *N. crassa* was 74.0 U/ g dry substrate under the same conditions.

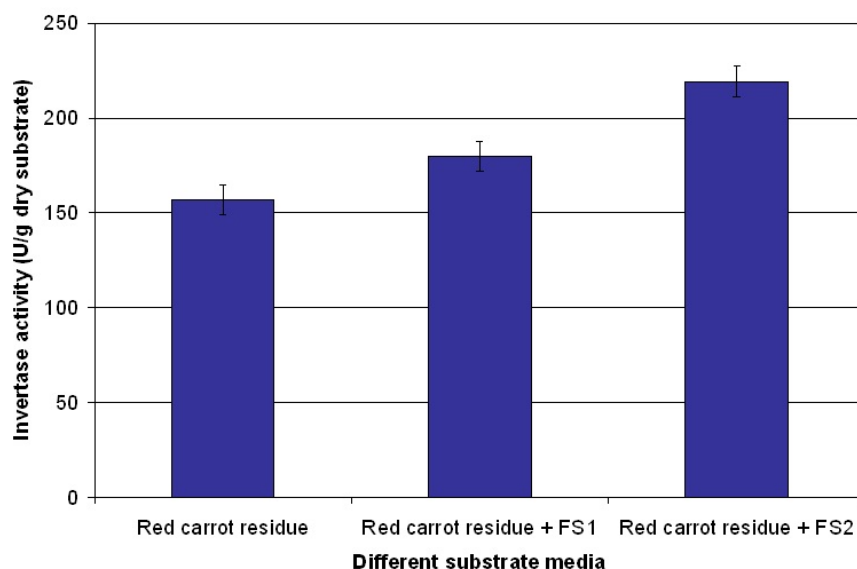


**Fig. 1:** Estimation of extracellular invertase produced by *S. cerevisiae* cultivated on different food processing wastes by SSF.

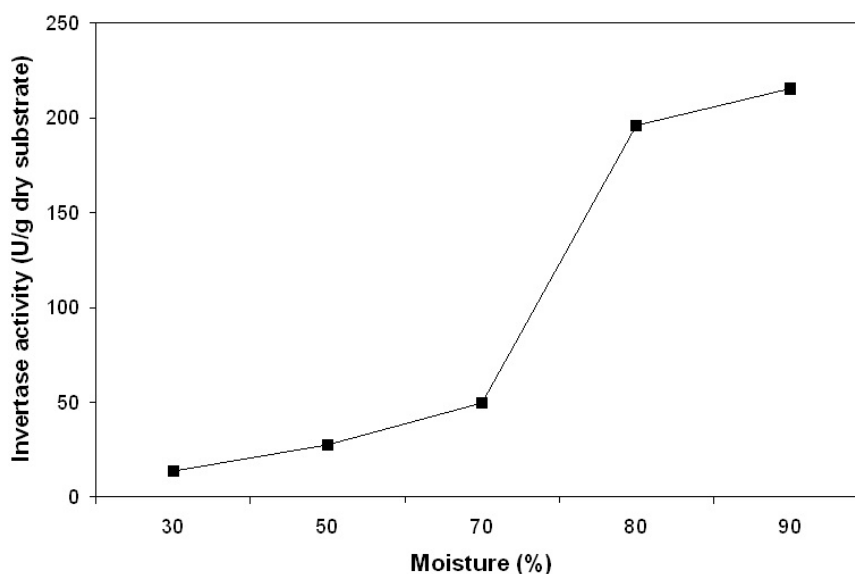
Chemical analysis of the dry Egyptian red carrot residue revealed that it contains 38.5 g % carbohydrate, 5.2 g % fat, and 4.0 g % protein which suggested that it can be utilized for microbial growth. The moisture content was found to be 89.5 %, so it can serve as substrate for the production of enzymes using SSF technique. Krishna (2005) reported that numerous nutrients can regulate sporulation through metabolic effects in SSF. These nutrients include carbon and nitrogen sources, minerals, and vitamins or cofactors.

The yield of extracellular invertase produced by growing *S. cerevisiae* on red carrot residue inoculated with either feeding solutions (FS1 or FS2) after 3 days of incubation was illustrated in Fig. 2. It can be observed that using feeding solution (FS2) in the fermentation process gave higher yield of invertase (219.3 U/g dry substrate) compared to the control (157.0 U/g dry substrate). On the other hand, there was a slight increase in the enzyme production after using feeding solution (FS1) which contained only sucrose and yeast extract (180.0 U/g dry substrate). These results are in agreement with Ashokkumar *et al.* (2001) who reported that the productivity of extracellular invertase from *A. niger* by SSF was higher after optimization of the medium with seven components as presented in feeding solution (FS2).

In SSF, the existence of an optimum moisture content of the medium has been stressed as it has profound effects on growth kinetics, and on the physicochemical properties of solids, which in turn affects productivities (Lonsane *et al.*, 1992). So, the effect of different initial moisture contents on the production of the enzyme was studied (Fig. 3). It was found that the production of the enzyme by *S. cerevisiae* was significantly affected by initial moisture content of the culture. Invertase production was increased approximately 15 times by increasing the moisture content from 30 % to 90 % giving 215.5 U/g dry substrate. These results may be attributed to the explanation stated by Krishna (2005) that lower moisture tends to reduce nutrient diffusion,



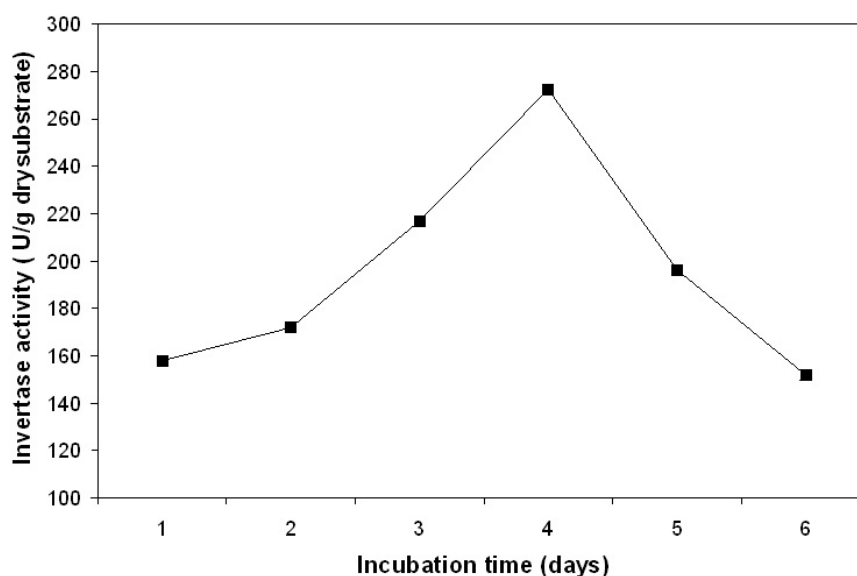
**Fig. 2:** Estimation of extracellular invertase produced by *S. cerevisiae* cultivated on red carrot residue using feeding solutions ( FS1 or FS2)



**Fig. 3:** Effect of initial moisture content on extracellular invertase production by *S.cerevisiae* cultivated on red carrot residue + FS 2.

microbial growth, enzyme stability and substrate swelling. Similar results were reported by Mamma *et al.* (2008) who found that the optimum moisture content for the production of invertase from different fungal strains by SSF using orange peels was 90%.

Another main parameter affects the productivity of the enzyme is the incubation period of growth. The effect of different incubation periods on the production of extracellular invertase by *S. cerevisiae* NRRL Y-12632 using red carrot residue and feeding solution (FS2) with 90% moisture of the substrate was demonstrated in Fig. 4. It can be observed that extracellular invertase reached a maximum activity after 4 days of incubation (272.5 U/g dry substrate), followed by a decline in the enzyme activity till the 6<sup>th</sup> day of incubation. Similar behavior was reported for extracellular invertase production from *A. niger* (Park and Pastores, 2003) and from *S. cerevisiae* (Rashad *et al.*, 2005). Lower incubation period (3 days) for both intra and extracellular *A. niger* invertase was reported by Sirisansaneeyakul *et al.* (2000).



**Fig. 4:** Effect of incubation period on extracellular invertase production by *S.cerevisiae* cultivated on red carrot residue + FS 2 with 90% moisture content.

The crude enzyme preparation was obtained by conducting fermentation processes under the optimal culture conditions tested in the present study. The whole optimum culture media obtained from several batches were collected and the culture filtrate was concentrated to be used in the trials of getting the enzyme in a pure form.

Partial purification of the crude enzyme was carried out by fractional precipitation using either acetone or ammonium sulfate but the results indicated unsuitability of both for obtaining active enzyme with higher yield (data not shown).

It was preferable to load the concentrated culture filtrate directly into anion exchange chromatographic column (DEAE-cellulose) (Table 1). In this step, a large part of the contaminating proteins was removed and it was found that extracellular invertase was eluted from the ion exchanger with two fractions, the first one (FI) was eluted at 0.1 M NaCl having 216.5 U with a yield of about 72 % and purification fold 6.8, and the second one (FII) was eluted at 0.2 M NaCl having 63.0 U with a yield of 21 % and purification fold 2.59. The active fractions eluted at 0.1 M NaCl (FI) were pooled, dialyzed and concentrated by lyophilization and applied on Sephacryl S-300 (Table 1). One peak having invertase activity was separated having specific activity of 2350 U/mg protein with a yield of about 5% and purification fold about 29. The result of enzyme recovery was less than those obtained by Nguyen *et al.* (2005); Rashad *et al.* (2006) and Guimaraes *et al.* (2009). On the other hand, the purification fold obtained was much better than those obtained by Rubio *et al.* (2002); Rashad *et al.* (2006) and Guimaraes *et al.* (2009).

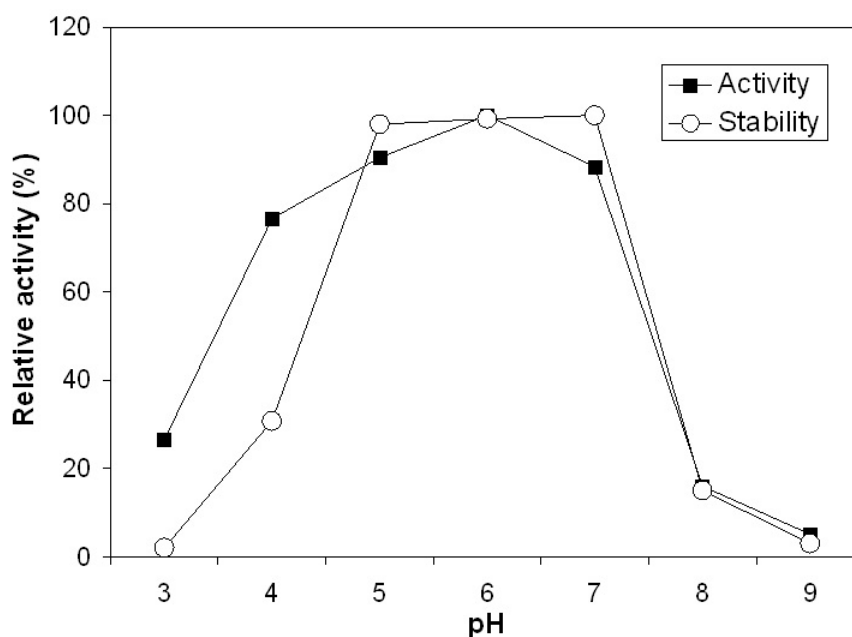
**Table 1:** Purification of extracellular *S. cerevisiae* invertase.

Purification steps	Total activity (units)	Protein(mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	300.0	3.7	81.1	1.00	100.00
DEAE-cellulose					
0.01M Phosphate buffer	1.30	0.09	14.44	0.18	0.43
0.1 M NaCl fraction (FI) *	216.5	0.39	555.1	6.8	72.2
0.2M NaCl fraction (FII)	63.01	0.3	210.03	2.59	21.0
Sephacryl S-300	14.1	0.006	2350	28.98	4.7

\* 0.1 M NaCl fractions were collected and applied to Sephacryl S-300 column.

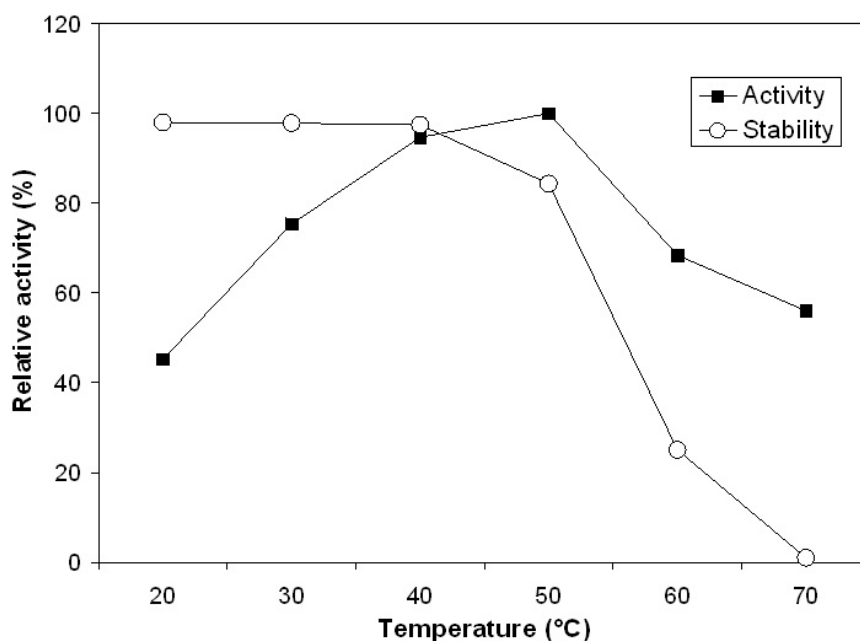
Some properties of the pure extracellular invertase were studied. The results in Fig. 5 demonstrated a pH optimum of 6.0 which was close to the pH optimum of *A. niger* invertase (5.5) as described by Nguyen *et al.* (2005). The optimum pH value was higher than those reported by many authors (Quiroga *et al.*, 1995; Rubio *et al.*, 2002; Rashad *et al.*, 2006 and Guimaraes *et al.*, 2007 & 2009), while it was lower than that reported by Vorster and Botha (1998) who found that the optimum pH of neutral invertase was 7.2.

Extracellular purified invertase was found to be stable at pH 5.0 – 7.0 (Fig. 5) which was closer to the results of Nguyen *et al.* (2005) who found that *A.niger* invertase was stable in the pH range from 5.0 to 6.5. 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.



**Fig. 5:** Effect of pH on the activity and stability of pure extracellular *S.cerevisiae* invertase.

Based on Fig. 6, the enzyme shows maximum activity at 50 °C and lost its activity rapidly when measured at temperatures higher than 50 °C. This result was similar to that reported by Nguyen *et al.* (2005) who found that the optimum temperature of *A.niger* invertase was 50 °C. Higher values of optimum invertase temperatures were reported by many authors (Rubio *et al.*, 2002 ; Guimaraes *et al.*, 2007& 2009 and Hussain *et al.*, 2009), while lower value (30°C) was reported by Rashad *et al.* (2006).



**Fig. 6:** Effect of temperature on the activity and stability of pure extracellular *S.cerevisiae* invertase.

The thermal stability of the pure extracellular invertase in the absence of substrate was examined at six different temperatures (20 – 70°C) (Fig. 6). Full activities of the enzyme were retained up to 40 °C for 1h and about 85% of the residual activity was retained at 50 °C. The activity decreased rapidly when the enzyme was

incubated at temperature higher than 50 °C. The enzyme stability was in the same range of invertases as illustrated by Quiroga *et al.* (1995), while it was lower than those from *R. glutinis* (Rubio *et al.*, 2002), *A. niger* (Nguyen *et al.*, 2005), and *S. cerevisiae* (Rashad *et al.*, 2006) which were thermostable up to 60 °C.

The purified extracellular invertase was tested for substrate specificity. The enzyme was highly active towards sucrose (100%) and slightly active towards raffinose (14.7%) but it nearly did not show any activity towards trehalose, melezitose, maltose, sorbitol, inulin, and glycerol. These results suggest that the enzyme is a  $\beta$ -D-fructofuranoside fructohydrolase, able to attack the  $\beta$ -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 (*R. glutinis*; *S. cerevisiae* and *A. niveus* respectively).

The Michaelis constant ( $K_m$ ) value of the pure enzyme was found to be 60 mM while its  $V_{max}$  was 35.5 min<sup>-1</sup> mg<sup>-1</sup> protein as calculated by Hanes-Woolf plot. The rate of sucrose hydrolysis decreased by increasing substrate concentration, which may be due to substrate inhibition. The  $K_m$  value was similar to that obtained with the invertase from *A. niger* (Rubio and Maldonado, 1995) and seemed to be higher than those prepared from *A. ochraceus* (Guimaraes *et al.*, 2007) and *A. niveus* (Guimaraes *et al.*, 2009), while it was lower than those from *R. glutinis* (Rubio *et al.*, 2002) and *S. cerevisiae* (Rashad *et al.*, 2006).

The effects of metal ions on the activity of the purified extracellular invertase are shown in Table 2. The results indicated that the enzyme was completely inhibited by Hg<sup>2+</sup> at low concentration (1mM), while it was slightly inhibited by Ba<sup>2+</sup>, Zn<sup>2+</sup>, and Fe<sup>2+</sup> at the same concentration. On the other hand, a slight increase in the enzyme was noticed by using 1mM of Co<sup>2+</sup>. The inhibition of invertase by Hg<sup>2+</sup> was reported by many authors (Ghosh *et al.*, 2001; Rashad *et al.*, 2006 and Guimaraes *et al.*, 2009) and they suggested that thiol groups at the catalytic site are important for the invertase activity. Stimulation of invertase activity by Co<sup>2+</sup> was also reported by Rubio *et al.* (2002) and Rashad *et al.* (2006), while Nguyen *et al.* (2005) found that the enzyme was slightly inhibited by addition of 1mM Co<sup>2+</sup>.

**Table 2** : Effect of various metal ions on the activity of extracellular *S.cerevisiae* invertase

Compound (1mM)	Relative activity (%)
Control (none)	100
CuSO <sub>4</sub>	101
FeSO <sub>4</sub>	89.4
HgCl <sub>2</sub>	-ve
MgCl <sub>2</sub>	100
CaCl <sub>2</sub>	97.9
BaCl <sub>2</sub>	76.6
NiCl <sub>2</sub>	100
ZnSO <sub>4</sub>	88.3
CoCl <sub>2</sub>	109.3

The molecular weight of the pure extracellular invertase was found to be 95.5 KDa as determined by gel filtration technique on Sephacryl S-300. This result is similar to that obtained by Rubio and Maldonado (1995) who found that invertase from *A. niger* isolated from mouldy lemons had a molecular mass of 95 KDa according to gel filtration with Sephadex G-150. Higher molecular weights were observed by gel filtration for invertases of *R. glutinis* (Rubio *et al.*, 2002), *A. ochraceus* (Guimaraes *et al.*, 2007) and *A. niveus* (Guimaraes *et al.*, 2009). In contrast, lower values have been reported for some microorganisms, such as *A. niger* (Hirayama *et al.* 1989), *L. reuteri* (de Gines *et al.* 2000) and *S. cerevisiae* (Rashad *et al.*, 2006).

### Conclusion:

This study indicates the potential for the use of food processing wastes such as red carrot jam processing residue for the efficient production of invertase by *S. cerevisiae* NRRL Y-12632 in SSF, thereby resulting in recycling waste materials for conversion into useful product of higher value as a by-product.

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