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MICROBIAL PRODUCTION OF INVERTASE AND ITS UTILIZATION IN PREPARATION OF INVERTED SUGARS

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ABSTRACT

The present investigation was carried out to clarify the feasibility of invertase production from some agricultural wastes (chicory roots, lettuce leaves, grass, cabbage top, potato leaves and sweet potato leaves.) using *Aspergillus oryza* NRRL 451 and *Saccharomyas cerevisiae* CBS 1200. The optimal conditions for enzyme production and action were determined. The hydrolytic efficiency of enzyme for hydrolysis sucrose to produce inverted sugar was studied. Also the physicochemical properties of syrups produced were studied.

Results indicated that invertase is successfully produced by *Aspergillus oryzae* $NRRL_{451}$ and *Saccharomyces cerevisiae* CBS 1200 using all tested substrates as carbon sources. The maximum production of invertase (5.31 and 2.79 units/ml medium) were recovered by cultivating *Aspergillus oryzae* $NRRL_{451}$ and *Saccharomyces cerevisiae* CBS 1200 on a media contains 2.5 and 3% grass powder at pH 5.0 and 5.5 after 72 and 48 hours of fermentation at 30°C respectively. The maximal activity of enzyme was at pH range 5.0 to 5.5 and 45 to 50°C, while the enzyme was stable at pH values from 4.5 to 7.0 and below 50°C. It was inhibited by Ag and Hg. Invertase produced by *Aspergillus oryzae* $NRRL_{451}$ has high hydrolytic efficiency (95.22%) and it could be recommended for production of inverted sugar on commercial scale.

Keywords: agricultural wastes, invertase, *Aspergillus oryza*, *Saccharomyces cerevisiae*, inverted sugar.

INTRODUCTION

Inverted sugars are used extensively in carbonated beverages, canning, bread baking, glazed fruits and numerous other products. Ninety percent invert syrup in bulk was used by large bakers and confectioners (Schneider, 1970 and Junk and Pancoast, 1973).

Production of soluble sugars on sweeteners by enzymatic hydrolysis of some by-products could make a significant contribution to the overall production of food and energy from renewable resources (Webb *et al.,* 1986).

Invertase (B-D- fructofuranosidase, EC.3.2.1.26) is produced microbiologically using different species of fungi, i.e. *Aspergillus Oryzae* and *Aspergillus niger* by (Annuniziato and Mahoney, 1987 and Wallis *et al.,* 1997). Dealing with yeasts, several investigators used *Saccharomyces cerevisiae* to produce invertase (Mansfeld *et al.,* 1995; Narciandi *et al.*, 1995 and Vitolo *et al.*, 1995). Besides, *Azotobacter chroococcum* bacteria was used by De-La-Vega *et al.*, (1991) for production of invertase. Chen 1995 and Abd El-Hady, 1999 examined the effect of the initial sucrose concentration on the invertase production in shaking batch cultures of *Aspergillus Japonicus* and *Aspergillus niger.* They found that the enzyme production increase with prolonging the time at different sucrose concentrations and the maximal enzyme production

reached upon increasing the sucrose concentration up to 20%(w/v) and then decrease. Inverted sugars are used in a large scale in Egypt on confectionary factories (Taiseer, *et al.*, 1986).

This present of work was carried out in order to study the use of *Aspergillus Oryzae* NRRL 451 and *Saccharomyces Cervisiae* CBS 1200 for the production of invertase from some agricultural wastes. Some enzyme kinetics, efficiency of sucrose inversion to inverted sugar were studied.

MATERIALS AND METHODS

Materials:

Raw Materials (Agricultural wastes)

Chicory roots (*Cichorium intybus L.*). Lettuce leaves (*Lactuca Sativa L.*), Grass (*Lolium Perenne*), cabbage top (*Cirsum aleraceum*), Potato leaves (*Solanum Tuberosum L.*) and Sweet potato leaves (*Impomea batata L.*) were collected from fields of Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt after harvesting. The samples were washed with tap water, then cut into small pieces and dried in an air oven at 60-70°C and milled in a mill, then sifted in 100 mesh screen sieve. The recovered powder was kept in polyethylene bags and stored at room temperature until using.

Microorganisms:

Aspergillus Oryzae 451 and *Scaccharomyces Cerevisiae* CBS 1200 were obtained from type culture collection of the Agricultural Chemical Technology Institute, Technical University, Budapest, Hungary. The organisms were maintained on 2% Jerusalem artichoke tuber extract and 1.5% agar slants.

Medium and cultivation:

Enzyme was produced using the procedure of Baysal *et al.*, (1994), which modified by adding the prepared agricultural wastes powder in a concentration of 1% of the medium composition. The medium was also contained 0.23% $NH_4 NO_3$, 0.37% $(NH_4)_2 HPO_4$, 0.1% KH_2PO_4, 0.05% Mg SO_4, 0.15% yeast extract, the initial pH was 5.0. Medium was autoclaved at 121°C for 30 min. Spores and yeast cells were suspended with 0.001% Tween 80, 1 ml of spore suspension (10^7 spores /ml) and 1 ml of cell suspension (10^8 cells/ml) was used to inoculate 100ml flasks containing 25ml medium. Incubation was at 30°C for 60 hours on a shaking incubator at 150 rpm.

Crude enzyme:

At the end of incubation period, the medium filtrate was separated from the mycelium and cells, then the filtrate was used as the crude enzyme solution throughout the experiments for enzyme assays and hydrolysis of sucrose.

Invertase activity:

The enzyme activity was determined according to method of Baysal *et al.*, (1994). The reaction mixture contained 1ml of 1% sucrose in 0.1M sodium acetate buffer pH 5.0 and 0.1ml crude enzyme. The mixture was held at 50°C for 5 min. The amount of reducing sugar was determined by 3,5-

dinitrosalicylic acid method (Miller, 1959). One unit (U) of enzyme activity was defined as one micromole of reducing sugar produced per minute.

Properties of invertase:

The effect of pH and temperature on invertase activity and stability were assayed according to the method described by (Haraguchi *et al.*, 1990).

Testing hydrolytic activity of invertase:

To accomplish the application, it was necessary to evaluate the hydrolyzing efficiency of two enzyme produced by *Aspergillus Oryzae* NRRI 451 and *Saccharomyces Cerevisiae* CBS 1200 on the hydrolysis of slurries containing 20% pure sucrose as a substrate with enzyme concentration of 2 units/ml according to the method described by Abd El-Hady (1999). The hydrolyzing period was prolonged for 10 hours at 45°C in shaker flasks containing 25ml of slurry with initial pH 5.0. The conversion was calculated according to the following equation:

$$\% \text{ Conversion} = \frac{\text{Reducing sugar (mg/ml)} \times 0.95}{\text{Initial substrate concentration (mg/ml)}} \times 100$$

Characteristics of inverted liquid sugar:

Inverted liquid sugar (syrup) produced by enzymatic hydrolysis of sucrose at the optimum conditions was neutralized by sodium bicarbonate and concentrated to 80% solids by vacuum evaporator at a temperature of 45 to 55°C. The final product was stored in a glass container.

Physico-Chemical properties of syrups produced by enzymatic hydrolysis of sucrose:

Specific gravity, refractive index, total solids and total soluble solids of syrups were determined according to the A.O.A.C. (1990). Viscosity was determined as described by Collins *et al.*, (1977) method. Color index was determined by measuring the optical density at 420nm. As described by Taiseer *et al.*, (1986) pH value was measured according to the method outlined by Gross (1967). Titratable acidity was performed using Abou-shady (1998).

Statistical analysis:

The data were analyzed statistically using the analysis of variance and the means were further tested using the least significant difference test (LSD) as outlined by Steel and Torrie (1980).

RESULTS AND DISCUSSION

Factors affecting invertase activity (type and concentration of carbon source, fermentation period and pH of media) were studied.

1. Effect of carbon source on enzyme activity.

Data shown in Table (1) represent the effect of carbon sources on the activity of invertase produced by *Asp. Oryzae and S. Cerevisiae*.

Data showed that grass powder is the best carbon source. The maximum enzyme activity was obtained in grass media (2.51 and 0.95

unit/ml) for invertase produced by *Asp. Oryzae and S. Cerevisiae* respectively. This could be due to the partial hydrolysis of grass fructan to fructose which was used as a good carbon source by the microorganism (Muller and Steller, 1995). Besides, no significant difference was noticed between invertase activity produced in grass media and that produced in chicory roots media. However, the minimum invertase activity was observed in cabbage top media (1.82 and 0.63 unit/mL for both microorganisms, respectively) and it was significantly different from the enzyme activity obtained in both grass and chicory roots media.

Table (1). Effect of the different sources on invertase activity.

Carbon source (1% of media)	Enzyme activity (unit/ml medium)*	
	Enzyme produced by *Aspergillus oryzae* NRRL$_{451}$	Enzyme produced by *Saccharomyces cerevisiae* CBS 1200
Chicory roots	2.18±0.41 a	0.85±0.07 a
Lettuce leaves	1.95±0.08 ab	0.69±0.04 ab
Grass	2.51±0.24 a	0.95±0.02 a
Cabbage top	1.82±0.11 b	0.63±0.06 b
Potato leaves	2.03±0.14 ab	0.71±0.07 ab
Sweet potato leaves	2.11±0.18 ab	0.78±0.05 ab

Production of invertase was carried out at pH 5 and 30°C for 60 hours at 150 rpm.
Each value was an average of three determinations.
*** Mean±SE**
Values followed by the same letter in column are not significantly different at P≤0.01.

2. Influence of grass powder concentration on enzyme activity.

Grass powder was used in concentrations varied from 0.5% to 5% of media composition, to determine the best concentration for producing the highest invertase activity (Table 2).

Data reveal that the enzyme activity increased significantly with increasing concentration of grass powder up to 2.5% and 3% of media used for growing *Asp. Oryzae and S. cerevesiae*, respectively. Meanwhile, any further increase of grass powder concentration caused significant decrease of enzyme activity. At the optimum grass powder concentration, the enzyme activity produced by *Asp. Oryzae and S. cerevesiae* was 4.75 and 2.32 unit/mL, respectively. Baysal *et al.*, (1994) found that with high substrate concentration, accumulation of free reducing sugars at the initial hours (0-24h.) of fermentation is observed. Easily available sugars probably cause catabolite repression and consequently less enzyme activity is observed.

3. Effect of fermentation period on enzyme activity.

Fermentation was carried out in a grass powder media (2.5% and 3% for *Asp. oryzae and S. cerevesiae,* respectively) at pH 5 and 30°C at 150 rpm. Data reported in Table (3) indicated that the maximum invertase activity (5.25 units/ml) was obtained after fermentation period of 72 hours using *Asp. Oryzae*. However, a period of 48 hours was the optimum period for production the higher enzyme activity by *S. cerevesiae*. These results are nearly similar to the findings of Allais *et al.*, (1986) who found that the invertase activity was reached its maximum when the cell growth was in the stationary phase.

Table (2):Effect of using different concentrations of grass powder in media composition on the produced invertase activity.

Grass powder conc. in media (%)	Enzyme activity (unit/ml medium)*	
	Enzyme produced by *Aspergillus oryzae* NRRL$_{451}$	Enzyme produced by *Saccharomyces cerevisiae* CBS 1200
0.5	1.44±0.15 e	0.62±0.04 e
1.0	2.49±0.08 d	0.98±0.07 d
1.5	3.55±0.17 c	1.35±0.11 bc
2.0	4.73±0.13 a	1.65±0.15 b
2.5	4.75±0.18 a	2.07±0.22 a
3.0	4.52±0.14 a	2.32±0.18 a
3.5	4.00±0.12 ab	2.00±0.12 ab
4.0	3.62±0.19 bc	1.76±0.13 ab
4.5	3.18±0.14 c	1.50±0.08 b
5	2.72±0.22 d	1.20±0.06 c

Production of invertase was carried out at pH 5 and 30°C for 60 hours at 150 rpm.
Each value was an average of three determinations.
* Mean±SE
Values followed by the same letter in column are not significantly different at P≤0.01.

Table (3):Effect of incubation period on inverstase activity produced in grass media at pH 5 and 30°C at 150 rpm.

Fermentation period (h)	Enzyme activity (unit/ml medium)*	
	Enzyme produced by *Aspergillus oryzae* NRRL$_{451}$	Enzyme produced by *Saccharomyces cerevisiae* CBS 1200
12	1.73±0.24 e	0.85±0.12 d
24	2.41±0.31 d	1.53±0.18 b
36	3.15±0.38 c	2.18±0.21 ab
48	3.85±0.35 b	2.79±0.28 a
60	4.60±0.38 ab	2.25±0.30 ab
72	5.25±0.29 a	1.75±0.29 b
84	4.51±0.25 ab	1.12±0.18 c
96	3.89±0.30 b	0.65±0.20 d

Production of invertase was carried out at pH 5 and 30oC for 60 hours at 150 rpm.
Each value was an average of three determinations.
* Mean±SE
Values followed by the same letter in column are not significantly different at P≤0.01.

4. Effect of media's pH on enzyme activity.

Effect of pH on invertase activity produced by *Asp. oryzae and S. cerevesiae*, in optimum grass media at 30°C was studied. The enzyme activity was measured at various pH from 3.0 to 7.0. Data presented in Table (4) declare that the optimal pH for invertase produced by *Asp. Oryzae* was 5.0, since it showed enzyme activity of 5.31 units/ml. However pH value of 5.5 was found to be the optimal pH for enzyme obtained by *S. cerevesiae*. At these conditions the enzyme activity was 2.79 units/ml. In all cases, there was no significant difference between invertase activities at pH values from 5.0 to 6.0.

Table (4):Effect of pH values on invertase activity produced in grass media at 30°C at 150 rpm.

Medium pH	Enzyme activity (unit/ml medium)*	
	Enzyme produced by *Aspergillus oryzae* NRRL$_{451}$	Enzyme produced by *Saccharomyces cerevisiae* CBS 1200
3.5	3.30±0.37 c	0.73±0.14 d
4.0	4.05±0.30 b	1.65±0.20 c
4.5	4.80±0.28 ab	2.40±0.28 ab
5.0	5.31±0.25 a	2.75±0.30 a
5.5	5.19±0.22 a	2.79±0.27 a
6.0	5.00±0.31 a	2.72±0.25 a
6.5	4.80±0.34 ab	2.30±0.18 b
7.0	4.52±0.27 b	2.00±0.16 b

Production of invertase was carried out at pH 5 and 30°C for 60 hours at 150 rpm.
Each value was an average of three determinations.
*** Mean±SE**
Values followed by the same letter in column are not significantly different at P≤0.01.

Properties of the invertase crude enzyme.

1. Effect of pH on the enzyme activity.

The enzyme activity was measured in buffers at various pH from 3.0 to 8.0 at 30°C. As show in Fig (1), the enzyme showed maximal activity at pH 5-5.5.

2. Effect of temperature on the enzyme activity.

The enzyme activity was assayed at various temperature from 30 to 65°C at pH 5. As shown in Fig (2), the maximal activity was obtained at 45-50°C.

3. pH stability

The enzyme solution was kept at various pH from 3.0 to 8.0 at 15°C for 24 hr. Residual enzyme activity was measured. As shown in Fig (3), the enzyme was stable in the pH 4.5 to 7.0, but it decreased bellow pH 4.0 and over 7.5 *Asp. niger* enzyme are reported to have pH stability in the range of (4-7) at 30°C **(Nakamura et al., 1978)**.

4. Thermal stability

To investigate thermal stability, the enzyme solution was incubated at various temperature from 30°C to 65°C for 10min. at pH 5.0. As shown in Fig (4), the enzyme was stable below 50°C, while increasing temperature more than 50°C caused a thermal instability of the enzyme.

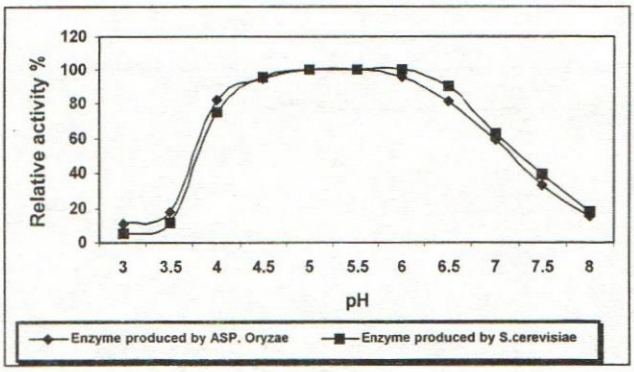

Fig. (1): Effect of pH on invertase activity

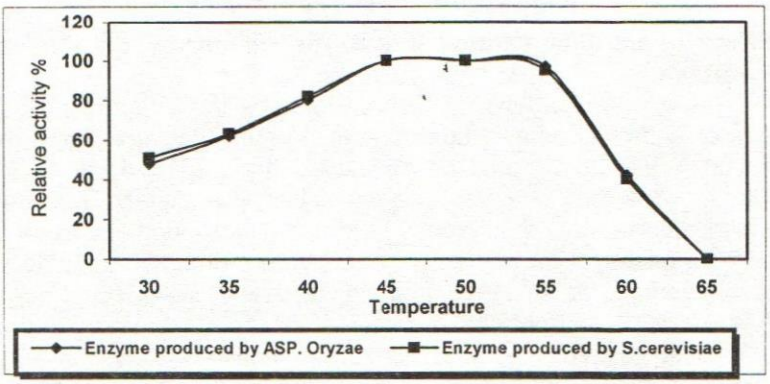

Fig. (2): Effect of temperature on invertase activity

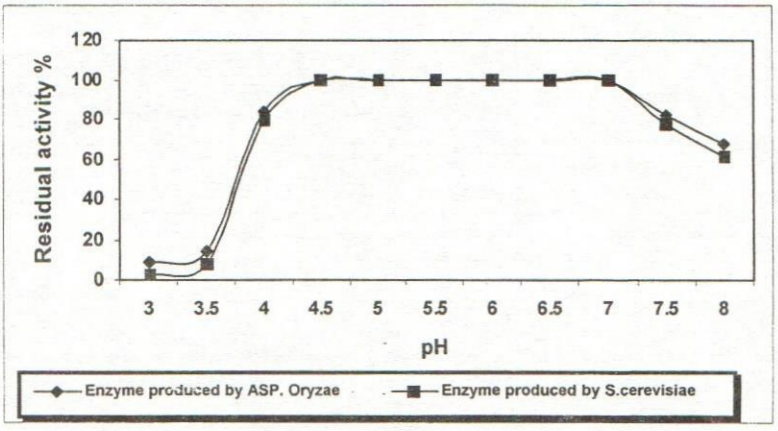

Fig. (3): pH stability of invertase activity.

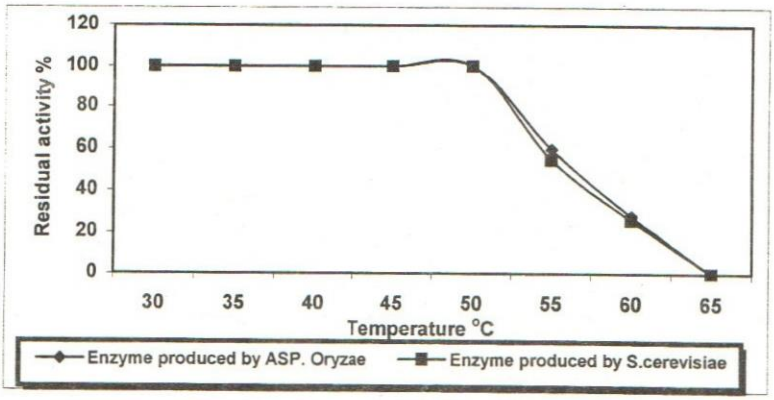

Fig. (4): Thermal stability of invertase.

5. Effect of various cations and some chemicals on the activity of invertase

This experiment was carried out to study the effect of adding various cations or chemical compounds as activators or inhibitors. From the results shown in Table (5) it could be concluded that Ag^{+} and Hg^{2+} are severe inhibitors, since they inhibited enzyme activity completely. This observation was also reported by other researchers and it was suggested that some-SH groups are essential for the activity of enzyme produced by molds and some yeast (Nakamura *et al.*, 1978 and Ettalibi and Baratti, 1990). Some other cations, i.e. AL^{3+} and pb^{2+} have partial inhibitory effect. Sodium chloride has no effect on enzyme activity. However, addition of Barium chloride or pyridoxine increased enzyme activity, so both of them could be considered as invertase activators (Nakamura *et al.*, 1978).

Table (5): Effect of various cations and some chemicals on the relative activity of invertase.

Compound	% Relative * activity	
	Enzyme produced by *Aspergillus oryzae* $NRRL_{451}$	Enzyme produced by *Saccharomyces cerevisiae* CBS 1200
Control	100	100
NaCl	100	100
$CaCl_2$	87	84
$ZnCl_2$	97	93
$CuSo_4$	93	92
$FeCl_3$	92	91
$AlCl_3$	68	66
$BaCl_2$	108	102
$Pb(NO_3)_2$	45	42
$MnCl_2$	72	73
$AgNO_3$	00	00
$Hg\ (CH_3COO)_2$	00	00
Pyridoxine	110	107
Sodium azide	78	79

All cations and chemical, were mixed with enzyme solution at 10^{-3} M concentration and preincubated at pH 5 and 30°C for one hour at 150 rpm.

*** % of the initial activity**

Hydrolytic efficiency of invertase:

Data shown in Table (6) indicated that invertase obtained from *Asp. Oryzae* has higher hydrolytic efficiency than that of enzyme produced by *S. cerevisiae*. The optimum conversion period for *Asp. oryzae* enzyme was 6 hours where 95.22% of the initial sucrose of the medium was converted. However, enzyme produced by *S. cerevisiae* required 8 hours to hydrolyze 85.11% of initial sucrose. The results obtained by Mejias and Perez (1996) and Abd-El-Hady (1999) support our findings. So, invertase produced by *Asp. oryzae* could be recommended for production of inverted sugars on commercial scale.

Table (6): Effect of using 2 units/ml of invertase on the hydrolysis of 20% sucrose during 10 hours at pH 5, 45°C and 150 rpm.

Time (h)	Enzyme produced by *Asp. Oryzae*		Enzyme produced by *S. Cervisiae*	
	Inverted sugar (mg/ml)	% hydrolysis	Inverted sugar (mg/ml	% hydrolysis
0	00.00	00.00	00.00	00.00
1	87.79	41.70	103.91	49.35
2	111.39	52.91	113.26	53.80
3	135.22	64.33	124.17	58.98
4	159.57	75.79	133.91	63.60
5	181.07	86.00	144.27	68.53
6	200.46	95.22	156.29	74.23
7	186.23	88.46	168.63	80.10
8	171.72	81.56	179.18	85.11
9	156.40	74.29	164.92	78.33
10	141.53	67.22	147.79	70.20

Physicochemical properties of syrups produced by enzymatic hydrolysis of sucrose

Data presented in Table (7) was indicated that the physicochemical properties of syrups produced by *Asp. oryzae* and *S. cerevisiae* enzymatic hydrolysis of sucrose. It is clear from the given results that total soluble solids (TSS%) were almost the same in all of the tested syrups (80%). Also, the results showed that the specific gravity, refractive index, viscosity, color index, pH and titrable acidity were nearly similar.

Table (7): Physico-chemical properties of syrups produced by enzymatic enzymatic of sucrose.

Syrup	% TSS	Specific gravity	Refractive index	Viscosity (centi-poise)	Color index	PH	Titratable acidity g/100g dry matter
* Syrup 1	80	1.435	1.519	21894	0.70	4.90	0.42
** Syrup 2	80	1.430	1.508	21882	0.71	5.01	0.45

* Syrup produced by *Asp. oryzae* enzyme hydrolysis from sucrose

** Syrup produced by *S. Cerevisiae* enzyme hydrolysis from sucrose Mean ± SE.

In conclusion, the inverted liquid sugar (syrup) produced by enzymatic hydrolysis on a large scale could be used in various food products as canneries, confectioneries and bakeries.

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إنتاج الأنفرتيز ميكروبياً والإستفادة منه فى تحضير السكر المحول

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**** قسم تكنولوجيا الأغذية – كلية الزراعة بكفر الشيخ – جامعة طنطا**

أجريت هذه الدراسة بهدف ايضاح إمكانية إنتاج الإنزيمات المحللة للسكروز (إنزيم الأنفرتيز) من بعض المخلفات الزراعية (وهي جذور الشيكوريا وورق الخس والعشب ورأس الكرنب وأوراق البطاطا وأوراق البطاطس) بواسطة فطر من جنس *Aspergillus oryzae* وخميرة من جنس *cerevisiae Saccharomyces*. وتضمنت الدراسة الظروف المثلي لإنتاج الإنزيم وخواصه، وأيضاً القدرة التحليلية للإنزيم المنتج علي تحليل السكروز بهدف إنتاج السكر المحول. وأيضاً تم دراسة الخواص الفيزوكيمياوية للشراب الناتج.

وقد أظهرت نتائج الدراسة أنه تم إنتاج إنزيم الأنفرتيز بنجاح باستخدام جميع المخلفات المختبرة كمصدر للكربون في بيئة الإنتاج. ووجد أن أعلي إنتاج لإنزيم الأنفرتيز هو (٥,٣١، ٢,٧٩ وحدة إنزيمية/مل بيئة) للفطر والخميرة عندما كانت بيئات الإنتاج تحتوي علي ٢,٥ و ٣% مسحوق عشب علي Hp ٥، ٥،٥ بعد ٧٢ و ٤٨ ساعة من التخمر علي درجة حرارة ٣٠٥ م علي التوالي. ووجد أيضاً أعلي نشاط للإنزيم الناتج من الفطر والخميرة كان عند pH من ٥ – ٥،٥ ودرجة حرارة من ٤٥–٥٥ م وبينما ثبات نشاط هذا الإنزيم كان علي pH من ٤,٥–٧ ودرجة حرارة أقل من ٥٥م . ووجد أيضاً أن الإنزيم الناتج يثبط بأيونات الفضة والزئبق وجد أن القدرة التحليلية لإنزيم الأنفرتيز المنتج بواسطة فطر *Aspergillus oryzae* كانت عالية ٩٥,٢٢% لذلك يوصي بإستخدامه في إنتاج السكر المحول علي نطاق تجاري.