Isolation and partial purification of invertase from different baker's and distillery *Saccharomyces cerevisiae*

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Abstract

Invertase enzyme from different commercial kinds of Baker's and distillery *Saccharomyces cerevisiae* was isolated and partially purified as a crude enzyme by salt stress process and ethanol precipitation. The lyophilization takes place for the enzyme isolated from activated distillery *S. cerevisiae*. The activities of partially purified invertase isolated from the different five baker yeasts under study ranged between 975 and 1801 IU/ml/min. While the activities of enzymes isolated from distillery yeasts before and after activation were 120 and 1100 IU/ml/min. The activity of lyophilized partially purified powder of enzyme isolated from activated distillery yeast was19300 U/g. The K_m and V_{max} values of the enzyme isolated from activated distillery yeast were 7.56 mg/ml and 294.12 IU/ml/min, respectively. These results were compared to those values of invertase isolated from Egyptian active dry Baker's yeast and the commercial invertase data sheet with a sample of sweetase 20 p enzyme obtained from Germany stern enzyme company. The results recorded in this paper clearly appeared that the active powder invertase could be obtained from activated distillery yeast by the easy and inexpensive method.

Key words: Invertase; Partial purification; Distillery yeast; Saccharomyces cerevisiae

Introduction

Saccharomyces cerevisiae is a most famous and important species of yeasts. It is perhaps the most useful yeast, having been instrumental to baking and brewing since ancient times. It is believed that it was originally isolated from the skin of grapes and it consider as one of the most intensively studied eukaryotic model organisms in molecular and cell biology (**Badotti et al.,**

2008). *Saccharomyces cerevisiae* produces an extracellular β-d-fructofuranoside fructohydrolase (invertase) when grown on a medium containing beta-fructofuranosides sucrose or raffinose, indicating that synthesis issubjected to induction by the substrate (**Stefanini et al., 2012)**.

Invertase (E.C.3.2.1.26) is an enzyme widely used in the food and drink industry. Enzymatic hydrolysis of sucrose by invertase results in the cleavage of α-1, 4-glycosidic bonds of sucrose, resulting in the formation of an equimolar mixture of glucose and fructose, known as invert sugar. The later is sweeter and easier to incorporate in industrial preparations than is granular sucrose (**David et al., 2006; Milovanovic et al., 2007).** Invert sugar is widely used in the production of non-crystalizing creams, jams, artificial honey and in confectionary industry and to a lesser extent in the industrial production of liquid sugar (**Emregul et al., 2006)**. Invertase also catalyzes transfer reactions with other acceptors beside water, resulting in the formation of oligosaccharides consisted of units of glucose and fructose **(Vicente, 2000)**.Also, this enzyme can be used for enhancing ethanol production from molasses by increasing invert sugar formation. The enzyme invertase has been found in bacteria, fungi, insects, mammals and vegetables, but the main industrial source is the yeasts. Yeast invertase has a molecular weight of 270 kDa and an isoelectric point between 3.4 and 4.4 and does not require cofactors for its activation (**Vicente, 2000; Isik et al., 2003; David et al., 2006)**.

On the other hand, several factories in Egypt are devoted forthe production of ethanol alcohol for industrial purposes using molasses through alcoholic fermentation processes. These fermentations are carried out by ethanol-tolerant strains of *Saccharomyces cerevisiae.* During ethanol production by fermentation, substantial amounts of yeast cells are produced

as a by-product of the fermentation processes. These large amounts of yeast cells are then further processed and dried at high temperature and sold as fodder yeast for animal feed. The annual production of fodder yeast by-product exceeds 6000 tons annually in Egyptian distillery factories and sold remarkably low prices (**Fadel and Foda, 2001)**.

In this study, scientific and experimental attempts were conducted to isolate and partial purify invertase from distillery yeasts as an additive value in ethanol production in Abo-Qurqas factory and compering the characters of the isolated enzyme with invertase isolated from the Baker's yeasts and commercial invertase.

Materials and Methods

Materials

All chemicals were of analytical grade and obtained from Sigma Chemical Company. Baker' yeast of *Saccharomyces cerevisiae* produced under a commercial names: El-Tayeb active dry baker' yeast produced by Egyptian sugar industry and integrated company (ESIIC), Aladdin´s (made in Turkey), Majest (made by China angle yeast company in Benisuef, Egypt), Ferment (made in Turkey) and Turbo (made in Egypt by L.S.E. company in new Noubariya city) in addition to distillery yeast produced in Abo-Qorqas distillery factory, El-Minia, Egypt were collected and used in this study for isolation and partial purification of invertase. Commercial invertase under product name of sweetase 20 p was obtained as gift from commercially available source of industrial grade named Stern Enzym GmbH and Co. KG, Germany derived from *Saccharomyces cerevisiae* which have activity as 20000 U/g, pH stability 3.0 - 6.0, and thermal stability 40 – 80°C.

Activation of distillery yeast

The distillery yeast was collected after anaerobic ethanol fermentation in Abo- Qurqus factory and washed by distilled water for five times in order to remove ash and ethanol content. Then re-suspended in 1 % (w/v) sucrose after final washing and allowed to grow for 24 hrs, then cultivated in 500 ml conical flask. Each flask contains 100 ml of sterile medium composed of (g/L): sucrose, 100; di-ammonium phosphate, 4 and yeast extract, 3. Then flasks were incubated for 24 hrs at 35ºC. The growing yeast was collected by centrifugation and used for invertase isolation by salt stress process.

Enzyme Isolation

Invertase was isolated from Baker and distillery yeasts by the method described by **Scope (1982)** and **Andjetkovic et al. (2010)** with some modification as follow: *Saccharomyces cerevisiae* cells (30 g) were suspended in deionized water (1:1 w/w). Toluene (3%) and 120 ml of a 0.1 M aqueous solution of sodium bicarbonate were added. The suspension was incubated for 24 hrs at room temperature allowing cell-autolysis to occur. The crude cell extract was cooled in an ice-bath and centrifuged for 20 min at 5,000 x g and 4°C as crude invertase. The pH in supernatant was adjusted to 4.0 with 1 M sulphuric acid and allowed to sediment overnight at 4ºC. The sediment was removed by centrifugation and the crude enzyme solution was stored at 4°C.

Precipitation of Invertase with Ethanol

Invertase was enriched and concentrated from the initial extract using one- step method of precipitation with ethanol at 4ºC (**Andjetkovic et al., 2010**). Cold 96% ethanol was added to cold fresh crude isolated enzyme

(1:1). The pellets was obtained by centrifugation after short period in ice, then completely dissolved in small amount of 0.1M acetate buffer, pH 4.8 prepared by addition 2.3 ml of glacial acetic acid and 4.92 g of sodium acetate in 1L and dialyzed overnight at 4ºC against deionized water. Material precipitated during dialysis was removed by centrifugation as previously mentioned. The obtained supernatant was representing partial purified invertase (**Ward, 2012**).

Lyophilization

The ethanol precipitated protein was collected and lyophilized by lyophilizer (Vir Tis, Model 6KBTES-55 Lyophilizer at Assiut University Mycological Centre, Egypt) into invertase powder enzyme.

Determination of Enzyme Activity and Concentration

A total of 100 µl of an enzyme solution of one g powder invertase in 10 ml buffer solution was added to 0.3 M sucrose solution in 50 mM acetate buffer (400 µl) at pH 5. After 5 min at 50ºC, the reaction was terminated by addition of 3, 5-dinitrosalicylic acid reagent (500 µl) and the mixture was boiled in water bath for 5 min. Before measuring absorbance at 540 nm, 4 mL of deionized water was added (**Bernfeld, 1955**). The standard curve was obtained with different concentrations (between 0.5 and 10 mM) of an equimolar mixture of D-glucose and D-fructose in 50 mM acetate buffer at pH 5.0. One unit of the invertase activity (U) corresponds to the amount of enzyme that catalyzes the hydrolysis of one µmol of sucrose per one min under described assay conditions. Invertase concentration (total protein) was determined calorimetrically at 546 nm. The intensity of the color formed is directly proportional to the amount of proteins present in the sample by using bovine albumin standard kits as the following equation (**Gornall et al., 1949; Ryan and Chopra, 1976**).

$$\text{Total Proteins in g/dl} = \frac{\text{(Abs.) sample}}{\text{(Abs.) standard}} \times 6$$

Determination of Enzyme Kinetic Constants

The kinetic constants: K_m (Michaelis-menten constant) and V_{max} (maximum reaction velocity) values of the lyophilized partial purified invertase were determined by measuring enzyme activity at different concentrations of sucrose. The reaction mixture at each sucrose concentration contained the same amount of invertase. K_m value of invertase for sucrose was determined using the Lineweaver-Burk plots various sucrose concentration against invertase activity in each assay in the reaction mixture (Lineweaver and Burk, 1934) as the following equation:

$$\frac{1}{v} = \left(\frac{Km}{Vmax}\right)\frac{1}{[s]} + \frac{1}{Vmax}$$

Where v= velocity and [s] = substrate concentration

Results and Discussion

1- Isolation and Partial Purification of Enzyme

Invertase from different commercial kinds of Baker's and distillery *Saccharomyces cerevisiae* was isolated and partially purified. It was isolated by using salt stress on yeast cells for 24 hrs then centrifugation was done to obtain crude invertase. The crude enzyme was then precipitated by cold ethanol 96 % and centrifuged under cold conditions. Finally, lyophilization of the ethanol precipitate enzyme was carried out for the enzyme which isolated from activated distillery *S. cerevisiae* to obtain the enzyme in a powder form. The enzyme activities, protein contents and specific activities of the crude and ethanol precipitated invertase were determined and the data are shown in Table (1) and illustrated in Figure (1).

The results in Table (1) indicated that the total activities of crude enzyme isolated using salt stress from the different five Baker's yeasts (El-

Tayeb, Aladdin´s, Majest, Ferment and Turbo Baker's yeasts) were ranged between 68250 and 126070 U, while the total activities of partial purified invertase using ethanol precipitation from the same five kinds of Baker's yeasts were ranged from 54030 to 101100 U. The reduction in enzyme activities by ethanol precipitation step reached to 18.17, 21.14, 18.79, 19.81 and 20.84% of the crude enzyme activities in cases of El-Tayeb, Aladdin´s, Majest, Ferment and Turbo Baker's yeasts, respectively. The highest active invertase was that isolated from Ferment yeast (made in Turkey), while the lowest active enzyme was isolated from Turbo yeast (made in Egypt by L.S.E. Company in new Noubariya city) as shown in Table (1) and illustrated in Figure (1). Also, the total contents of protein were reduced by ethanol precipitation and recorded as 863.1, 855, 858, 966 and 738 mg in comparison with the initial content of protein isolated by salt stress which were 2492, 2506, 2423, 2772 and 2249 mg in cases of El-Tayeb, Aladdin´s, Majest, Ferment and Turbo Baker's yeasts, respectively. However, the specific activities of the invertase extracted from the five kinds of Baker's yeasts after ethanol precipitation were 74.73, 101.75, 89.51, 104.66 and 73.21 U/mg comparing with 31.63, 44.02, 39.02, 45.48 and 31.45 U/mg at the initial liquid, respectively. It was also observed that invertase after partial purification was purified to almost 2.36, 2.31, 2.29, 2.30 and 2.33 fold, respectively (Table, 1).

In this respect, **Aslam et al. (2013)** precipitated invertase of *S. cerevisiae* by ammonium sulfate and found that the total enzyme activity reduced to 34016 (64 % of the initial enzyme activity), total protein reduced to 210 mg/ml from 404 initial protein and specific activity was 162 U/mg comparing with 131U/mg at the crude broth being 1.2 fold pure. **AL- Sa'ady (2014)** isolated invertase from three *Saccharomyces cerevisae* of different sources (China, Turkey and Egypt) and reported that the yeast from China

had high specific enzyme activity reached to 34.7 U/mg, while the activities of enzyme isolated from Turkey and Egypt were only 15.5 and 26.7 U/mg, respectively.

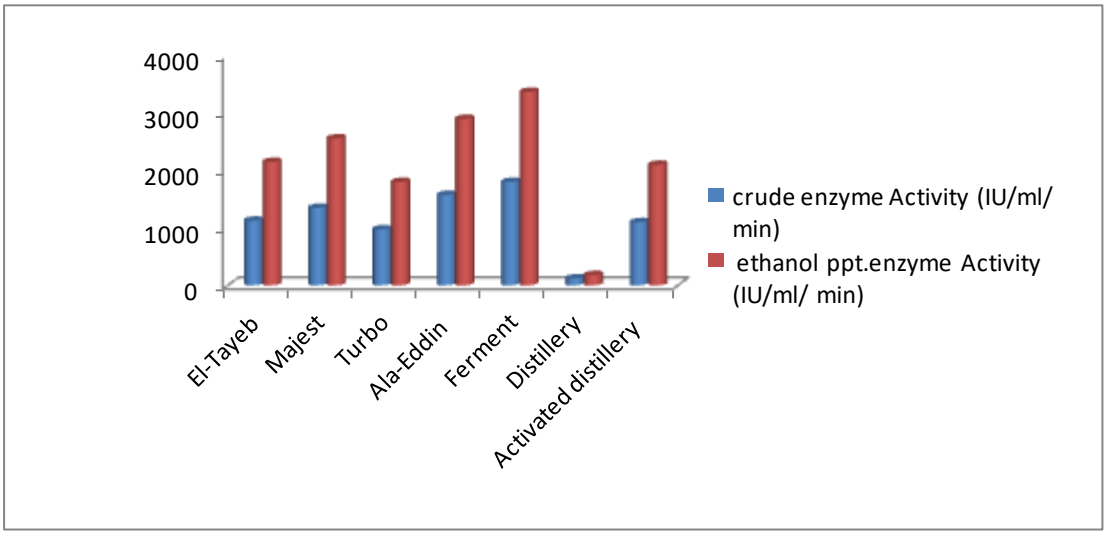
On the other hand, the partial purification of invertase from distillery yeast before and after activation process using ethanol precipitation led to reduce the total enzyme activity from 8400 to 1800 and 77000 to 63000 U, respectively (Table, 1). From these results, it was clear that the activity of crude invertase isolated from distillery yeast before activation (8400 U) was very low compared with the activity of crude enzyme isolated from activated distillery yeast (77000 U) as shown in Table (1) and illustrated in Figure (1). Also, the reduction percent of ethanol precipitated enzyme activity of invertase from distillery yeast before activation reached to 78.57 % compared with only 18.19 % in case of activated distillery yeast. It is worth to mention that the activity of partial purified invertase using ethanol precipitation from activated distillery yeast (63000 U) was nearly equal to those from El-Tayeb active dry Egyptian Baker yeast (64500 U) as in Table (1) and Figure (1). However, the specific activities of the invertase extracted from distillery yeasts before and after activation were found to be 18.15 and 73.94 U/mg comparing with 8.57 and 31.16 U/mg at the initial liquid, respectively. It was also indicated that invertase after partial purification from distillery yeasts before and after activation were purified to almost 2.12 and 2.37 fold, respectively (Table, 1).

A total of 9.5 g lyophilized powder (with protein content reached to 380 mg / g) produced from each 100 g of activated distillery yeast. The activity of lyophilized powder invertase of the activated distillery yeast was192 IU/min/ml of an enzyme solution of one g powder invertase in 10 ml buffer solution. This activity was 1920 U/ml and 19300 U/g.These activity values were relatively equal to those recorded by invertase isolated from

Egyptian active dry Baker yeast (El-Tayeb) by the same method, which were 1940 U/ml and 19400 U/g. These values were nearly similar to those recorded in the commercial invertase data sheet with sample of sweetase 20 p enzyme obtained from Germany stern enzym company which equal to 20000 U/g. In this respect, nearly similar result was recorded by **Aslam et al. (2013)**. They reported that the total activity of purified extracellular invertase from *Saccharomyces cerevisiae* reached to 20110 U/g.

Table 1 : Characterization of crude and ethanol precipitated invertase from different yeast sources.

Yeast sources	Steps	Volume (ml)	Enzyme Activity (IU/ml/ min)	Total activity (U)	Protein (mg/m)	Total protein (mg)	Specific activity (U/mg protein	n-fold	Yield %
El-Tayeb Baker's yeast (Egypt)	Crude Invertase	70	1126	78820	35.60	2492	31.63	1.0	100
	Ethanol precipitation	30	2150	64500	28.77	863.1	74.73	2.36	81.83
Majest Baker's yeast (Egypt)	Crude Invertase	70	1351	94570	34.62	2423	39.02	1.0	100
	Ethanol precipitation	30	2560	76800	28.60	858	89.51	2.29	81.21
Turbo Baker's yeast (Egypt)	Crude Invertase	70	975	68250	31.00	2249	31.45	1.0	100
	Ethanol precipitation	30	1801	54030	24.60	738	73.21	2.33	79.16
Ala-Eddin Baker's yeast (Turkey)	Crude Invertase	70	1576	110320	35.80	2506	44.02	1.0	100
	Ethanol precipitation	30	2900	87000	28.50	855	101.75	2.31	78.86
Ferment Baker's yeast (Turkey)	Crude Invertase	70	1801	126070	39.60	2772	45.48	1.0	100
	Ethanol precipitation	30	3370	101100	32.20	966	104.66	2.30	80.19
Distillery yeast (Egypt)	Crude Invertase	70	120	8400	14.00	980	8.57	1.0	100
	Ethanol precipitation	10	180	1800	9.92	99.2	18.15	2.12	21.43
Activated distillery yeast (Egypt)	Crude Invertase	70	1100	77000	35.30	2471	31.16	1.0	100
	Ethanol precipitation	30	2100	63000	28.40	852	73.94	2.37	81.81



Figure(1): Crude and ethanol precipitation enzyme activity.

2- Determination of invertase kinetic constant(Km and Vmax)

The kinetic parameters (K_m and V_{max}) were determined at 50°C and pH 5.0 for lyophilized partially purified invertase isolated from activated distillery yeast at sucrose concentrations which ranged between 1 to 20 mg/ml as substrate (=2.9 – 58.5 mM). The 1/V and 1/S values were recorded in Table (2). The K_m and V_{max} were calculated as 7.56 mg/ml and 294.12 IU/ml/min. The kinetic parameters of enzymatic reaction were calculated by the Lineweaver -Burk linearization using the Michaelis- Menton kinetic model (Table, 2 and Figure, 2).

K_m value of invertase isolated from activated distillery yeast in this study (7.56 mg/ml =22.11mM) is nearly similar to those recorded by **Chavez et al. (1997).** They reported that the invertase isolated from *Saccharomyces cerevisiae* exhibited K_m values 25 mM. **Ribeiro and Vitolo (2005)** calculated the K_m of soluble and insoluble invertase and found that the K_m for soluble enzyme was 18.3 mM and that for insoluble enzyme was 29.1 mM. On the other hand, K_m value of invertase in the present study was comparatively higher than those recorded by other researchers for invertase isolated from other sources (**Vorster and Botha, 1998; Amin et al., 2010).** The former

reported that the K_m values of sugarcane invertase was 9.8, while the second found that the K_m values of sugarcane invertase was 5 mM. Also, **Hatch et al. (1963) and Mahbubur-Rahman et al. (2004)** reported that the K_m value of sugarcane invertase was 8 mM. The kinetic parameters K_m and V_{max} ofinvertase from *Saccharomyces cerevisiae* MK were determined by **Shankar et al. (2014)** and were recorded as 0.3410 mg/ml and 0.5953 µm/min/mg, respectively. **Uma et al. (2010)** reported K_m of 0.23 mg/ml and V_{max} of 15.8 U/mg for invertase of *Aspergillus flavus*. **Almeida et al. (2005)** investigated the kinetic parameters of auto-immobilized enzyme and recorded that the K_m and V_{max} of the enzyme were 447 mM and 2,805 mmol/min, respectively. **Uma et al. (2012)** also calculated K_m and V_{max} of invertase and found that the K_m was 0.26 mg/min and V_{max} was 28.57 U/mg. Whereas, **Mona and Mahman (2009)** evaluated the invertase from *Saccharomyces cerevisiae* NRRL Y-12632 and recorded 60 mM as K_m while its V_{max} was 35.5 min / mg protein.

Table 2 : Determination of 1/V and 1/S for partially purified invertase Isolated from activated distillery yeast

(s) substrate (sucrose) concentration mg/ml	Velocity(v) (IU/ml/min)	1/V	1/S
1	39.66	0.0252	1.00
2	49.45	0.0202	0.50
3	57.46	0.0174	0.33
4	84.38	0.0119	0.25
5	103.79	0.0096	0.20
6	115.69	0.0086	0.17
7	143.45	0.0070	0.14
8	145.56	0.0069	0.13
9	178.89	0.0056	0.11
10	191.54	0.0052	0.10
11	202.51	0.0049	0.09
12	209.77	0.0048	0.08
13	219.39	0.0046	0.08
14	229.52	0.0044	0.07
15	235.00	0.0043	0.07
16	236.27	0.0042	0.06
17	243.02	0.0041	0.06
18	243.02	0.0041	0.06
19	243.02	0.0041	0.05
20	243.02	0.0041	0.05

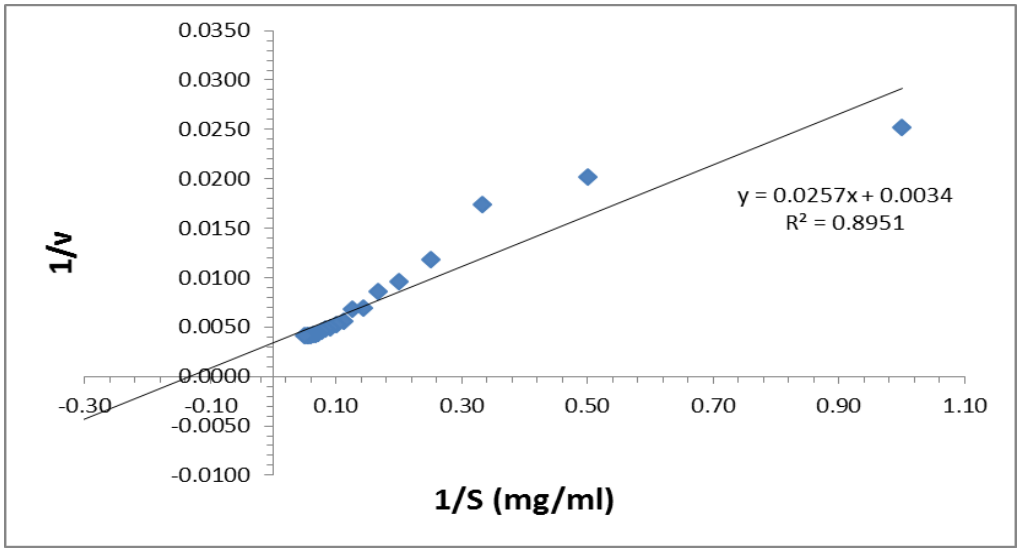


Fig . (2): Determination of Km and Vmax of partially purified invertase isolated from activated distillery yeast

Conclusion

In the present study, different commercial kinds of baker and distillery yeasts were selected for invertase isolation as crude solution. Then partially purified protein precipitated by cold ethanol and dried to powder form by lyophilization process. The results appeared that the all types of Baker's yeasts under study have considerable different concentration of invertase can be isolated and partial purified by cold ethanol as precipitated agent. The lyophilized partially purified invertase isolated from activated distillery yeasts had activity reached to 19300 U/g with K_m and V_{max} values of 7.56 mg/ml and 294.12 IU/ ml/min, respectively. Moreover, lyophilization of the partial purified enzyme into a powder form was a very useful step because it enable us to reduce the enzyme volumewhile keeping the enzyme fully activity.

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الملخص العربي

فصل وتنقية انزيم الانفرتيز جزئيا من انواع مختلفة من خمائر الخباز والتقطير

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قسم النبات و الميكروبيولوجي- كلية العلوم جامعة أسيوط

معهد دراسات وبحوث تكنولوجيا صناعة السكر لشئون الدراسات العليا والبحوث

إدارة الرقابة والجودة بمصنع تقطير ابوقرقاص – شركة السكر والصناعات التكاملية المصرية

إدارة الرقابة والجودة – مصانع سكر ابوقرقاص - شركة السكر والصناعات التكاملية المصرية

تم في هذا البحث الفصل والتنقية الجزئية لإنزيم الانفرتيز من خمسة انواع تجارية مختلفة من خمائر الخباز الجافة النشطة والمنتشرة في السوق المصري وكذلك خميرة التقطير المنتجة في مصانع التقطير التابع لشركة السكر والصناعات التكاملية المصرية. وقد سجل إنزيم الأنفرتيز المفصول من خمس أنواع مختلفة من خمائر الخباز التجارية المنتشرة في السوق المصري نشاطا تراوح ما بين 1801 , 975 وحدة دولية لكل مللي في الدقيقة بينما سجل الانزيم المفصول من خميرة التقطير قبل وبعد عملية التنشيط لها نشاطا قدر 1100 , 120 وحدة دولية لكل مللي في الدقيقة علي التوالى. تم حساب قيمة كلا من ثابت ميشيل (K_m), السرعة القصوي (V_{max}) للإنزيم المفصول من خميرة التقطير المنشطة وكانت تساوي 7.56 مجم / مليليتر و 294.12 وحدة دولية لكل مللي في الدقيقة على التوالى. هذا وقد تم كذلك مقارنة تلك النتائج مع عينة من انزيم الانفرتيز تم الحصول عليها من المانيا انتاج شركة (stern enzym) وإنزيم الإنفرتيز المفصول من خميرة الخبارالجافة النشطة المصرية المنتجة بواسطة شركة السكر والصناعات التكاملية المصرية وأثبتت الدراسة أنه يمكن الحصول علي إنزيم الإنفرتيز في صورة بودرة جافة نشطة وذات فاعلية عالية من خميرة التقطير النشطة والمنتجة بمصانع أبو قرقاص التابعة لشركة السكر والصناعات التكاملية المصرية.