PRODUCTION OF *Aspergillus niger* INVERTASE *ENZYMES ON MEDIA CONTAIN SOME AGRO-INDUSTRIAL SUGAR BEET CROP BY-PRODUCTS*

Nariman, O.A. Youssef*; Sahar R. Abd El-Hady and Aboshady, Kh.A.***

* *Sugar Crops Res. Inst., Agric. Res. Center, Giza, Egypt.*

** *Food Tech. Dept. Fac. of Agric. Kafr El-Sheikh Univ.*

ABSTRACT

Choice of most suitable concentration of beet pulp, beet leaves and beet molasses for production active invertase enzymes by *Asp. niger* mold was performed. Testing the hydrolytic activity of produced enzymes was also studied.

Results indicated that produced invertase enzymes achieved their maximum levels 189.3 and 133.2 unit/100 ml medium, when 2.5% beet pulp and 4% beet leaves were used as carbon source in production media. The biosynthesis of invertase enzymes by *Asp. niger* were also induced by about 2.3 and 3.7 folds in beet pulp and beet leaves media by addition 1% and 3% beet molasses by-product to such media, respectively. Dynamic study shows that maximum enzyme activities 231.2, 447, 155.5 and 517.5 unit/100 ml medium were recorded after 7^{th}, 5^{th}, 4^{th} and 6^{th} day of fermentation period using selected media containing 2.5% beet pulp, 2.5% beet pup + 1% beet molasses, 4% beet leaves and 4% beet leaves + 3% beet molasses, respectively. High hydrolytic activity of produced invertases indicated that the maximum conversion percent of 20% sucrose syrup were 86.75, 82.03% and for 40% sucrose syrup were 92.69%, 89.2% which occurred by invertases produced in beet pulp-molasses medium and beet leaves-molasses medium, respectively. Such converted syrups are considered as a source of fuel, food, chemicals, vitamins and multitude of other useful products.

INTRODUCTION

The problems of utilizing agro-industrial wastes as a raw materials for the production of chemicals are greatly simplified if they are first converted to glucose and other sugars that accomplished by either acid or enzymatic hydrolysis. These sugars can be used as microbial substrates to produce a variety of fermentation chemicals (alcohols, solvents… etc.) or single-cell protein or they can serve as the base for the manufacture of organic chemicals. In addition, the enzymatic process offers the advantage of being carried out at moderate reaction condition of pressure and temperature and eliminating problems of producing unwanted by-product or reversion compounds (Andren *et al.*, 1975).

Enzyme catalyzed synthesis of oligosaccharides has a number of attractions. However, most of the processes so far established at the laboratory scale have not been scaled up yet. This is partly because of the low yield, high cost of appropriate enzymes (especially the transferases) and suitable substrates. β-D-fructofuranosidase (invertase EC 3.2.1.26) and sucrose are well known, readily available and relatively in expensive, so they should be considered the ideal for large scale application (Somiari and Bielecki, 1995).

Fructo-oligosaccharides become of major interest because of their favorable functional properties. They are now produced commercially through the enzymatic synthesis from sucrose by microbial β-D-fructosyltransferases (Ftases, EC 2.4.1.9) or β-fructofuranosidases (invertases) (Fases, 3.2.1.26) with high transfeructosylating activities (Chen, 1995).

According to the previous considerations, the present study was designed to produce the active invertase using some agro-industrial sugar beet crop by-products (beet pulp and leaves) as carbon source in the production media of *Asp. niger* mold. Inducing effect of beet molasses on the production of invertase and the suitable fermentation period were determined. The hydrolytic efficiency of the obtained enzymes for producing invert sugars syrup from sucrose was also investigated.

MATERIALS AND METHODS

I. Materials:

Organism: *Aspergillus niger* mold used for invertases production was supplied by Department of Agricultural Chemical Technology, Faculty of Chemical Engineering, Technical University of Budapest, Hungary.

Raw materials:

1. Agro-industrial by -products:

Sugar beet pulp and beet molasses: were obtained from Delta Beet Sugar Company, Kafr El-Sheikh, Egypt. Beet pulp was well ground, mixed, packed and stored in polyethylene bags at room temperature. Beet molasses was packed in plastic jars and stored in a refrigerator at 4°C.

Sugar beet leaves: were collected from beet fields of Sakha Agricultural Research Station in Kafr El-Sheikh, Egypt. Sun dried for six days then milled in cereals machine mill and stored in polyethylene bags at room temperature until use.

2. **Corn steep liqueur:** was kindly obtained from Tura Starch Factory. Cairo, Egypt.

II. Method:

1. Production of invertase:

Preparation of stock culture and inoculum: Strain of *Aspgerigllus niger* was grown on slant potato dextrose agar (PDA) at 28°C for five days and kept at 4°C until use. The organisms were sub-cultured once a month. The medium of (PDA) was prepared as given in Difco (1974). Inoculum was prepared by transferring re-freshed spores of three slants by adding 10 ml sterilized distilled water to each slant, each slant was shacked vigorously for one minute, then added to 70 ml of 0.001% Tween 80 solution. One ml of the yielded suspension was used to inoculated 25 ml of the production medium.

Production of enzymes:

The medium used for invertases production was prepared according to the method described by Ongen-Baysal *et al.* (1994) that was modified by replacng the source of carbon by using the prepared by-products. The medium was composed of 0.23% NH_4NO_3, 0.37% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.15% yeast extract and mixed with selected amount of carbon sources i.e. sugar crop agro-industrial by-products. Additional modification was also applied by replacing the yeast extract by addition 0.565 gm of corn steep liquor (contains 2.6% N) as suggested by Abd El-Hady (1999). Replacing the yeast extract because it is considered very expensive for commercial production of enzymes (Chan *et al.*, 1991). The initial pH value of media was adjusted at 5.0. Experiments were carried out in 100 ml flasks containing 25 ml of medium that sterilized at 121°C for 30 minutes. The sterilized media were inoculated with 1 ml of spores suspension of *Asp. niger*, then incubated at 30° ± 2°C for five days on a shaking incubator at

150 rpm. The medium filtrate was separated from the mycelium after incubation and used as enzymes resource (Abd El-Hady, 1999).

2. Enzyme assay:

The enzyme activity was determined according to method of Ongen-Baysal *et al.* (1994). One ml of 1.0% sucrose in 0.1 M sodium acetate buffer (pH 5.0) was used as a substrate. Glucose-fructose standard curve was used for calculation units of enzymes. One unit of invertase activity was defined as the amount of enzyme which catalyzed the hydrolysis of one micromol of sucrose per minute (Barthomeuf *et al.*, 1991).

3. Testing hydrolytic activity of enzyme:

The hydrolysis was carried out in 125 ml flasks, containing 25 ml of mixture of enzyme filtrate solution and 0.1 M sodium acetate buffer solution containing 2 units/ml of enzymes and 20% or 40% sucrose (5 and 10 gms). Quarter ml of 1% sodium azide solution was added to inhibit the microbial growth during hydrolysis period. Flasks were incubated at 50°C on a rotary shaker at 120 rpm (Mandels *et al.*, 1981 and Mandels, 1982). Samples were taken after 1, 2, 3, 4, 5, 6, 7 and 8 hours of incubation time to estimate the release reducing sugar and the percent of conversion as described by Godbole *et al.* (1990). Glucose-Fructose (1: 1) standard curve from 0.125 to 1.75 mg sugars of mixture was used for calculation the converted sugar concentration. The percent of conversion was calculated according the following equation:

$$\% \text{ conversion} = \frac{\text{Reducing sugar}^* \text{ (mg/ml)} \times 0.9 \times 100}{\text{Initial substrate concentration (mg/ml)}}$$

* Reducing sugar was estimated by dinitrosalicylic acid (DNS) method as described by Miller (1959).

RESULTS AND DISCUSSION

1. Selection of suitable concentration of beet pulp and leaves for production of invertase enzyme:

Nine concentration were used to select the suitable concentration of beet pulp and leaves for producing the highest units of invertase by *Aspergillus niger*. The results in Table (1) indicated that invertase enzyme activity was increased with increasing beet pulp or leaves concentration in media and achieved their maximum values (189.3 and 133.2 units/100 ml medium) with

2.5% and 4.0% beet pulp and leaves concentration, respectively. Further increase of by-product concentrations caused a gradual repression of enzyme biosynthesis. Results in the same table also revealed that the maximum activities were obtained when the final pH values of the two media reached to 4.8 and 4.55, respectively.

Table (1): Effect of using different concentration of beet pulp and beet leaves on production of invertases by *Aspergillus niger* Mold.

By-products conc. % in media	Beet pulp medium		Beet leaves medium	
	Final pH of medium	Invertase activity (Unit/100 ml medium)	Final pH of medium	Invertase activity (Unit/100 ml medium)
0.5	4.8	142.4	4.50	19.4
1.0	5.4	153.7	4.90	21.2
1.5	5.3	154.6	5.20	21.5
2.0	5.0	158.9	4.90	26.5
2.5	4.8	189.3*	4.75	39.9
3.0	4.0	187.6	4.60	64.0
3.5	3.7	175.4	4.55	117.1
4.0	3.6	161.5	4.55	133.2*
4.5	3.3	151.1	4.45	122.6

Values are means of 3 determinations

Production conditions: Initial pH = 5, Incubation temperature 30°C ± 2°C and incubation period 5 days.

* Maximum activity of enzymes produced in media.

2. Inducing biosynthesis of *Asp. niger* invertase by addition of beet molasses to production media.

Beet molasses contain high ratios of carbohydrates, total nitrogen, minerals (ash) and some other contents (Youssif, 1996), it could be interesting to test it as inducing substrate for invertase production by *Asp. niger*. So it was added in seven concentration ranged from 0.5 to 3.5% to the selected media contain 2.5% beet pulp or 4.0 beet leaves, respectively.

The results presented in Table (2) indicated that the optimum concentration of beet molasses which induced the highest enzyme activity by *Asp. niger* were 1 and 3% for beet pulp and beet leaves media with final pH 5.2 and 6.0, respectively. At these levels of beet molasses and pH values, the biosynthesis of invertase enzymes was higher than controls without adding molasses by about 2.3 and 3.7 folds of the two used media, respectively.

It should be noted also from the same table, that further addition of beet molasses to each of by-products media caused a repression in the biosynthesis of invertases. Similar results were reported by Chan *et al.* (1991); Bokassa *et al.* (1993) and Abd El-

Hady (1999). They found that providing the medium with 3% of total reducing sugar (w/v) gave the maximum biosynthesis of invertases. They added that invertase activities were decreased upon increasing the molasses concentration in the medium.

Table (2): Effect of adding different concentration of beet molasses to the media contain the optimum concentration by-products on the production of invertase by *Aspergillus niger* Mold.

Beet molasses conc. % in media	2.5% Beet pulp medium		4.0% Beet leaves medium	
	Final pH of medium	Invertase activity (Unit/100 ml medium)	Final pH of medium	Invertase activity (Unit/100 ml medium)
0 (control)	4.5	191.0	4.4	132.0
0.5	4.9	390.0	5.0	359.9
1.0	5.2	445.0*	5.4	368.8
1.5	5.4	383.0	5.8	381.8
2.0	5.6	345.0	5.9	442.3
2.5	5.7	328.0	6.0	446.7
3.0	5.2	278.0	6.0	489.7*
3.5	5.2	233.0	6.1	468.8

Values are means of 3 determinations.

Production conditions: Initial pH = 5, Incubation temperature 30°C ± 2°C and incubation period 5 days.

* Maximum activity of enzymes produced in media.

3. Dynamic production of invertase enzymes by *Aspergillus niger* in different selected media:

For producing the highest yield of invertase enzymes by *Asp. niger*, the optimum period must be determined. So, this experiment was designed to figure out the kinetics of accumulation invertase enzymes in the different selected production media. It was carried out using four selected media containing optimum concentrations of tested by-products. Time course for each fermentation was followed until the maximal units of enzymes were accumulated in each medium, and no further increases could be detected.

Table (3) represent the kinetic behavior of enzymes production. The results revealed that invertases were start produced in all tested media after first day of incubation. The represented data indicate that invertase enzymes increased at a roughly constant rate after the first day of cultivation.

The maximum enzyme activities were reached after 7th, 5th, 4th and 6th days of fermentation period in beet pulp, beet pulp +

Table (3): Biosynthesis dynamic of invertase enzyme production in selected optimum media by using *Aspergillus niger* mold.

Fermentation period (days)	Used media							
	2.5% beet pulp medium		2.5 beet pulp + 1.0% beet molasses medium		4.0 beet leaves medium		4.0% beet leaves + 3.0% beet molasses medium	
	Final pH	Invertases activity unit/100 ml medium	Final pH	Invertases activity unit/100 ml medium	Final pH	Invertases activity unit/100 ml medium	Final pH	Invertases activity unit/100 ml medium
0	5.0	0.0	5.0	00.0	5.0	0.0	5.0	00.0
1	3.4	11.0	3.6	128.7	4.6	10.0	4.4	19.3
2	3.0	131.0	4.0	188.5	4.4	72.9	4.8	200.1
3	3.8	145.0	5.8	324.9	5.0	93.7	5.3	345.0
4	4.6	148.4	6.1	366.2	5.7	155.5*	5.9	418.8
5	4.9	190.0	6.4	447.0*	5.8	132.9	6.2	488.0
6	5.0	212.4	5.8	445.0	5.8	114.0	6.3	517.5*
7	5.1	231.2*	5.5	424.2	5.6	103.2	6.3	411.6
8	5.4	228.5	5.3	405.3	5.6	71.2	6.3	403.3

Values are means of 3 determinations

Production conditions: Initial pH = 5, Incubation temperature 30°C ± 2°C.

* Maximum activity of enzymes produced in media.

molasses, beet leaves and beet leaves + molasses media, in sequence. The obtained activities were 231.2, 447, 155.5 and 517.5 unit/100 ml medium of the previous mentioned media, respectively. On the other hand, in the early stage of cultivation, pH values fell from 5 to about 3, 3.6, 4.4 and 4.4 in the previous used media, respectively. Then pH began to rise gradually with increasing enzyme production.

4. Testing the hydrolytic activity of produced invertases on hydrolysis of sucrose syrups:

To accomplish the view of application, it was necessary to evaluate the hydrolyzing efficiency of produced invertase enzymes. In this relation, hydrolysis of syrups containing 20% and 40% pure sucrose as substrate with enzyme concentration of 2 units/ml were applied.

Results presented in Table (4) show that the hydrolysis time was done for 7 hours at 50°C in shacked flask containing 25 ml of slurry with initial pH 4.8. Maximum conversion (86.75% and 82.03%) of 20% sucrose syrup using beet pulp-molasses and beet leaves-molasses enzymes were obtained after 5 and 6 hours of hydrolysis. The yield of the recovered invert sugars were 192.77 and 182.29 mg sugar/ml mixture. In relation to 40% sucrose syrup, results in Table (5) indicate that the maximum hydrolysis of sucrose using beet pulp-molasses and beet leaves-molasses enzymes took place after 7 hours of incubation. The highest yield of invert sugars were 411.94 and 396.44 mg/ml slurry representing 92.69% and 89.2% of the initial concentration of sucrose, respectively. Our results are supported by findings of Godbole *et al.* (1990) and Amaya-Delgado *et al.* (2006). Accordingly, the profile data of hydrolysis show that tested invertases produced on media containing by-product of beet sugar crop had a high hydrolytic power. Also, it should be noted that, utilization of invert sugars as a source of fuel, food, chemicals, vitamins, single cell protein, and multitude of other useful products opens new vistas in the field of energy, food and chemicals to augment and conserve current world energy sources (Schlegel & Barnea, 1976 and Ester & Michele, 2007).

Table (4): Effect of two units invertases on saccharification 20% sucrose during seven hour of hydrolysis at 50°C and pH 4.8.

Saccharification period (hours)	The type of used enzymes			
	Invertases produced on beet pulp-molasses medium		Invertases produced on beet leaves-molasses medium	
	Converted sugars mg/ml	% conversion	Converted sugars mg/ml	% conversion
1	79.83	35.92	68.70	30.91
2	121.39	54.63	112.64	50.69
3	126.94	57.12	128.45	57.80
4	174.86	78.69	154.49	69.34
5	192.77	86.75*	175.53	78.99
6	187.75	84.49	182.29	82.03*
7	187.50	84.38	181.85	81.84

Values are means of 3 determinations

* The maximum conversion of sucrose %.

Table (5): Effect of two units invertase on saccharification 40% sucrose during eight hours of hydrolysis at 50°C and pH 4.8.

Saccharification period (hours)	The type of used enzyme			
	Invertases produced on beet pulp-molasses medium		Invertases produced on beet leaves-molasses medium	
	Converted sugars mg/ml	% conversion	Converted sugars mg/ml	% conversion
1	67.84	15.26	61.41	13.82
2	137.73	30.99	125.23	28.18
3	215.28	48.44	170.16	38.29
4	287.71	64.73	222.67	50.10
5	334.73	75.31	319.38	71.86
6	384.88	86.60	333.23	74.98
7	411.94	92.69*	396.44	89.20*
8	368.69	82.96	348.31	78.37

Values are means of 3 determinations

* The maximum conversion of sucrose %.

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إنتاج إنزيمات أنفرتيز فطر *Asp. niger* على بيئات غذائية تحتوى على بعض المخلفات الزراعية الصناعية لمحصول بنجر السكر

ناريمان عمر عبده يوسف* ــ سحر رمضان عبدالهادى ــ خالد على أبوشادى***

* معهد بحوث المحاصيل السكرية ــ مركز البحوث الزراعية ــ الجيزه ــ مصر

** قسم الصناعات الغذائية ــ كلية الزراعة ــ جامعة كفرالشيخ

الملخص العربى

تضمن هذا البحث اختيار أنسب التركيزات من مخلفات بنجر السكر الزراعية والصناعية بهدف إنتاج إنزيمات الأنفرتيز النشطة بواسطة فطر جنس *Asp. niger* ، كما تم اختبار القدرة التحليلية لهذه الإنزيمات.

أشارت النتائج إلى أن أقصى وحدات إنزيمية منتجة كانت ١٨٩,٣ ، ١٣٣,٢ وحدة إنزيمية/١٠٠ مل بيئة ، وذلك باستخدام بيئات غذائية تحتوى على ٢,٥% ، ٤,٠% من لب البنجر وأوراق نبات بنجر السكر كمصدر كربونى على التوالى. تم تحفيز إنتاج الإنزيمات على بيئتى لب البنجر وورق البنجر إلى حوالى ٢,٣ ، ٣,٧ أضعاف وذلك بإضافة ١% ، ٣% من مولاس البنجر لتلك البيئات على التوالى. وقد إتضح أن أعلى إنتاج لإنزيمات الأنفرتيز تم الحصول عليه كان ٢٣١,٢ ، ٤٤٧ ، ١٥٥,٥ ، ٥١٧,٥ وحدة إنزيمية/١٠٠ مل بيئة غذائية بعد اليوم السابع والخامس والرابع والسادس بالتنمية على بيئات لب البنجر ، لب البنجر + المولاس ، وورق البنجر ، ورق البنجر + المولاس على التوالى. قدرت أعلى قوة تحليلية لمحاليل السكروز بالإنزيمات المنتجة على أنسب البيئات المختارة (بيئة ٢,٥% لب بنجر + ١,٠% مولاس ، بيئة ٤% ورق البنجر + ٣% مولاس) حيث وصلت نسبة التحويل الإنزيمى لمحلول ٢٠% سكروز إلى ٨٦,٧٥% ، ٨٢,٠٣% ، ولمحلول ٤٠% سكروز إلى ٩٢,٦٩% ، ٨٩,٢% بإستخدام الإنزيمات المنتجة على البيئات الغذائية المختارة والمذكورة على التوالى. وتعتبر مثل هذه الأشربة المحولة مصدر من مصادر إنتاج الوقود والغذاء والكيماويات والفيتامينات إلى جانب كثير من المنتجات النافعة.