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ORIGINAL ARTICLES

Production and some characterization of ß-glucosidase from *Rhodotorula glutinis* isolate

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ABSTRACT

Collections of 13 yeast strains isolated from different sources of marine water (Ras Al Bar, Alexandria and Marsa Matruh) were screened for β-glucosidase production. *Rhodotorula glutinis was* found to be the best source of this enzyme production. The effect of medium composition and incubation conditions for fermentation process were established. The maximum β-glucosidase production (270units/ml) was occurred after 24h of incubation at 30°C on optimized medium containing cellobiose (2%) as a sole source of carbon and 0.5% $(NH_4)_2SO_4$ as a nitrogen source. The enzyme was found to be produced intracellular by the strain under study, therefore, physical and chemical treatments were applied to release the enzyme.The maximum enzyme release was observed when grinding with sterile sea sand (250 units/ml). The enzyme was partially purified by ammonium sulfate precipitation and the recovery was 75% of the total enzyme activity which affords 3.02 fold purification. The hydrolytic activity of the β-glucosidase was occurred at pH 6.5 and 45°C.

Key words: β-glucosidase, yeasts, identification, enzyme release, partial purification, enzyme properties.

Introduction

β-glucosidase or β-glucoside glucohydrolase [EC 3.2.1.21] has been widely used in ethanol production using various cellulosic agricultural residues such as corn stover, straw and bagasse (Pemberton *et al.,* 1980, Xin *et al.,* 1993 and Bothast and Saha1997) and in the synthesis of useful glucosidase (Shinoyama *et al.,* 1991 and Gunata *et al.,* 1994). In the flavor industry, β-glucosidases are also the key enzymes in enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermentation products (Guegen *et al.,* 1996). β-glucosidase is also useful in the process of deinking of printing ink from wastepaper (Krik and Jeffries 1996). Many bacteria, fungi and yeasts have been shown to produce β-glucosidase enzyme. Bacteria such as *Bretlanomyces bruxellensis* and *Oenococcus oeni* (Manasfield *et al*.,2001), fungi like *Aspergillus oryzae* (Riou, *et al*., 1998), *Fusarium oxysporum* (Christakopoulos *et al.,* 1995), *Trichoderma reesei* (Saloheimo *et al.,*2002), *Penicillium penophilus* (Ah-Ream *et al*.,2010), *Aspergillus niger* (Li-Chun Qiam *et al*.,2012)and the yeasts like *Candida peltata* (Saha and Bothast 1996) , *Candida curvata* (Sandhu *et al*., 1985) are also reported to be the main producers.

The objective of the present investigation was to optimize the cultural parameters for maximum production of β-glucosidase by *Rhodotorula glutinis* such as, effect of different carbon sources, nitrogen sources and sodium chloride concentrations on enzyme production has been studied. Furthermore, some properties of the enzyme were also investigated after partial purification.

Materials and Methods

Microorganisms:

Cellobiose utilizing yeasts were isolated from a variety of Egyptian coasts locations using a modified Dox agar medium containing cellobiose as the only source of carbon. All these isolates were screened for their β-glucosidase activity. The most active isolate was selected and identified according to Lodder (1970), Barnett and Pankhurst (1976).

Maintenance and culture conditions:

The selected *Rhodotorula glutinis* isolated from marine water sample obtained from Ras Al Bar coast was maintained on YNB agar medium at 30 °C and transferred to new slants every two months to keep it viable.

The medium used (Fenton *et al.,* 1982) contained (g/l) KH_2PO_4, 1.0., K_2HPO_4, 1.0 ., $(NH_3)H_2PO_4$, 1.0., $(NH_3)_2HPO_4$, 1.0., $MgSO_4$, 0.1., yeast extract 1.0 and distilled water 1000ml.The medium was supplemented

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with an appropriate carbon source at a concentration of 1.0 % (w/v) for estimation of yeast growth and β - glucosidase production.

Enzyme release treatment:

After the end of the incubation period (one day at 30°C), cells were harvested by centrifugation under cooling and the release of active enzyme was carried out by grinding of yeast cells with sterile sea sand. In addition, chemical treatments to release the enzyme were also carried out using organic solvents as toluene, n-butanol or t-butanol as described by Daroit *et al.,* (2007).

Enzyme assay:

β- Glucosidase assay was based on the procedure described by Riou *et al*., (1998). A 1-ml reaction mixture containing 5 mM *p*-nitro phenyl β- D-glucopyranoside (*p*NPG), 100 mM potassium phosphate buffer pH (7), and appropriate dilution of enzyme preparation. After incubation for 10 min at 30ºC, the reaction was stopped by addition of 2 ml of 1 M sodium carbonate and the liberated *p*-nitro phenol was monitored at 420 nm. One unit of β-glucosidase activity corresponds to the release of 1 µmol of *p*-nitro phenol min^{-1} under the assay conditions.

Enzyme purification:

The crude enzyme was subjected to different concentrations of ammonium sulphate precipitation. The precipitated fractions were collected by centrifugation at 6000 rev/min under cooling then dissolved in 0.05 M phosphate buffer pH 6.8 and dialyzed against the same buffer.

Protein determination:

Protein was determined by the method of Lowry *et al*., (1951) using bovine serum albumin as standard.

Effect of environmental variables on β-glucosidase production:

The effect of various carbon sources, different concentrations of the best carbon source, various nitrogen sources and different concentrations of NaCl on the production of enzyme was studied.

Characterization of β-glucosidase:

β-glucosidase activity was measured at different temperatures (30-60°C) and pH 3.6 – 8.0 by using different buffers. The buffers used were phosphate buffer (0.1 M pH5.6-8), sodium citrate (0.1 M pH 3) and sodium acetate (0.1 M pH 3.5-5.6). β-glucosidase activity was measured at 420 nm.

Substrate specificity:

Substrate specificity of the partially purified enzyme was determined by incubation of the partially purified enzyme in respective substrates (10 mM) at optimum temperature and pH for 10 min measuring the liberated *p*NP, glucose (Mukherjee and Khowala, 2002).

Results and Discussion

Screening test:

The ability of the isolated yeasts to produce β-glucosidase was investigated on a modified Dox medium containing amm.sulphate as a nitrogen source instead of its nitrogen (KNO_3) .Data in Table (1) indicate that isolate no. 5 supported the highest production of the enzyme after 24h of incubation at 30°C.

Identification of yeast isolate:

Taxonomical studies on the most promising isolate (i.e. isolate no.5) were shown in (Table 2 and Fig 1 a and b). The results indicated that this isolate could be identified as *Rhodotorula glutinis* according to Lodder (1970) and Barnett and Pankhurst(1976).

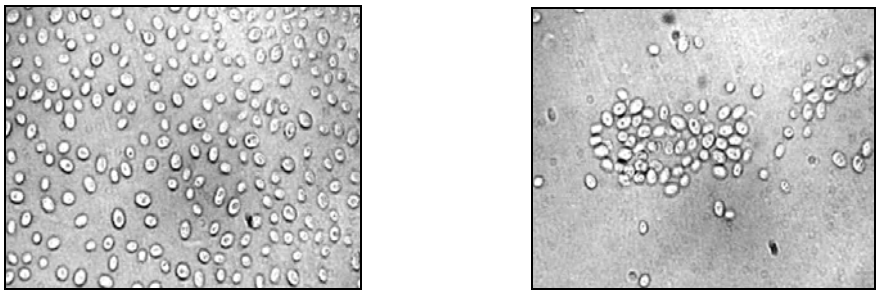
Table 1: Growth and β-glucosidase formation by the isolated yeasts.

Isolate No.	Growth O.D $_{600}$		Enzyme production (units/ml)	
	24h	48h	24h	48h
1	2.4	2.6	160	130
3	2.1	2.3	170	150
5	2.4	2.5	200	180
6	1.9	2.1	90	81
7	2.3	2.3	130	123
9	2.1	2.1	170	141
10	1.8	2.0	120	115
13	1.5	1.8	113	60

Table 2: Taxonomical studies on the most promising isolate.

Test	*Result*
COLONY MORPHOLOGY:	Orange to red. Colony is yeast like, soft, smooth, moist and sometimes mucous.
MICROSCOPIC EXAMINATION	On cornmeal-tween 80 agar at 25°Yeast, cells are round to oval (3.4 x 7.5) µm in diameter. A faint capsule is formed.
Germ tube test	-
Arthroconidia	-
Ascospores	-
Pseudohyphae	-
Urease	+
Assimilation of :	
Dextrose	+
Maltose	+
Sucrose	+
Lactose	-
Galactose	+
Melibiose	-
Cellobiose	+
Inositol	-
Xylose	+
Raffinose	+
Trehalose	+
KNO_3	+
Fermentation of :	
Dextrose	+
Maltose	-
Sucrose	-
Lactose	-
Galactose	-
Trehalose	-
Cellobiose	-

(-) = Negative (+) = Positive



A **B**

Fig. 1: A and B: Morphology of the most promising isolate.

Enzyme release treatments:

The industrial development of microbial intracellular enzymes has been hampered by the difficult and expense of releasing active enzyme from cells with a good yield(Lilly and Dunnill,1969). It is worthy to mention that β–glucosidase production by the chosen isolate was found to be intracellular. Therefore, physical treatments (grinding with sterile sea sand) and chemical treatments with organic solvents (toluene, n-butanol and t- butanol) were applied on yeast cells as recommended by Fenton *et al.,* 1982 and Selim *et al*., 1990, in order to

obtain its intracellular enzyme. The isolate was cultivated with shaking (150 rev/min at 30°C) in flasks containing Dox medium. After 24h of incubation, cells were harvested by centrifugation followed by resuspending in 0.1M Phosphate buffer.β–glucosidase was estimated in the clear filtrate of the growth medium and in the buffer after removing the treated cells. Results of physical and chemical treatments are shown in (Tables 3,4,5 and 6). Data obtained in Table 3 clearly indicate that grinding yeast cells with sterile sea sand in mortar under cooling was the most efficient method. The highest β-glucosidase release was detected after 45 min of grinding (250 units/ml). Furthermore, the prolonged grinding time decreased the enzyme activity.

Table 3: Effect of grinding with sterile sea sand on the rate of β-glucosidase release.

Treatment duration(min)	Enzyme activity (units /ml)
15	140
30	190
45	250
60	170

The results of enzyme release using organic solvents recorded in Tables(4,5 and 6) indicate that the solvent ratio (0.5 ml/ 0.2g cells) and contact time 30 min were the best treatments for enzyme release for all solvents used (toluene, n-butanol and t-butanol gave 200,180 and 165 units / ml) .Generally, The treatments with organic solvents were less efficient as chemical treatment compared with the physical treatment.

Table 4: Effect of toluene on the rate of β-glucosidase release from *Rhodotorula glutinis* cells.

Solvent Ratio ml/0.2g	Contact time/min	Enzyme release (units/ml) with different agitation time/h					
		0	1	2	4	8	24
0.5	15	14	160	182	140	130	110
	30	14	190	220	170	150	132
	60	14	170	191	158	140	122
1	15	14	143	170	135	118	102
	30	14	177	200	155	135	68
	60	14	130	153	128	112	52
1.5	15	14	125	148	132	121	77
	30	14	163	180	169	132	104
	60	14	118	128	115	76	61

Table 5: Effect of n-butanol on the rate of β-glucosidase release from *Rhodotorula glutinis* cells.

Solvent Ratio ml/0.2g	Contact time/min	Enzyme release (units/ml) with different agitation time/h					
		0	1	2	4	8	24
0.5	15	14	143	165	142	123	98
	30	14	155	180	151	115	102
	60	14	141	160	143	112	101
1	15	14	132	148	128	115	101
	30	14	148	163	133	119	109
	60	14	127	142	122	108	100
1.5	15	14	122	132	121	97	87
	30	14	138	154	132	102	98
	60	14	119	144	128	107	85

Table 6: Effect of t-butanol on the rate of β-glucosidase release from *Rhodotorula glutinis* cells.

Solvent Ratio ml/0.2g	Contact time/min	Enzyme release (units/ml) with different agitation time/h					
		0	1	2	4	8	24
0.5	15	14	136	149	131	110	82
	30	14	142	165	142	103	92
	60	14	128	159	130	98	87
1	15	14	125	132	128	102	91
	30	14	131	142	129	110	92
	60	14	119	123	102	89	76
1.5	15	14	112	120	102	88	65
	30	14	112	123	118	100	87
	60	14	102	112	101	89	60

The release of active enzymes from yeast cells using physical and chemical treatments was investigated by many authors and the results were varying according to the method was used and type of microorganism and enzyme. Selim *et al.,* (1990) found that β-glucosidase release in a good yield from *Kluyveromyces fragilis* NRRL Y-1137 by sonication followed in descending order by organic solvents, homogenization breakage and freezing and thawing, While Korish and Salem (2007) used homogenization with glass beads to release β-glucosidase from the yeast *Trichosporon* sp.

Effect of different carbon sources on the enzyme production:

Many carbon sources were tested separately in the medium. Results in (Table 7) show that both growth and enzyme production were maximally produced in the presence of maltose as only source of carbon (259 units/ml and OD $_{600}$ 2.9), followed by cellbiose (230 units/ml and OD $_{600}$ 2.5). Data in Table(7) clearly indicated that β-glucosidase was produced constitutively and cellobiose was the most efficient than maltose when compared as production ratio (92 and 89.3) by strain under study. Furthermore, cellbiose was used in the medium with different concentrations (0.5-3.0%w/v).The results in Table (8) showed that a notable increase of enzyme activity was correlated with the increase of cellbiose concentration reaching its maximum value (270 units/ml) when 2% final concentration of cellobiose was used, followed by a notable decrease at higher concentration. The results are agreement with that obtained by Selim *et al*.,(1989) and Korish and Salem (2007), they found that β-glucosidase was produced constitutively and cellobise, lactose and Maltose were the best carbon sourses for β-glucosidase production by *Kluyveromyces fragilis* and *Trichosporon* sp. respectively. While Saha *et al*.,1994 elucidated that corn bran followed by lactose , pullulan and cellobise were the best carbon sourses for enzyme production from *Aureobasidium pullulans*.

Table 7: Effect of different carbon sources on the production of β-glucosidase by a shaken culture of *Rhodotorula glutinis*.

Carbon source	Growth O.D $_{600}$	β-glucosidase activity	
		Units/ml	Production ratio
Glucose	2.7	160	59.3
Glycerol	2.1	102	48.6
Fructose	3.1	130	41.9
Galactose	1.9	100	52.6
Maltose	2.9	259	89.3
Sucrose	3.1	140	45.2
Sorbose	1.4	15	10.7
Cellobiose	2.5	230	92
Raffinose	1.3	5	3.8
Inulin	1.1	8	7.3
Starch	2.0	3	1.5

Production ratio explains the relation between units and growth.
Incubation time 24h at 30°C.

Table 8: Effect of different concentrations of cellobiose on the production of β-glucosidase by a shaken culture of *Rhodotorula glutinis*.

Concentration% (w/v)	Growth O.D $_{600}$	β-glucosidase activity (Units/ml)
0.5	2.3	210
1.0	2.5	230
1.5	2.6	250
2.0	2.4	270
2.5	2.1	210
3.0	1.6	150

Incubation time 24h at 30°C.

Effect of different nitrogen source:

Nine inorganic nitrogen sources were tested separately in the medium in such amount that the final concentration of N-base was not changed as that in the basal medium. Results in Table (9) clearly indicated that ammonium sulphate was the most suitable nitrogen sourse for β-glucosidase production (270units/ml) followed by ammonium phosphate mono basic (240 units/ml).The result was not agreement with that reported by Selim(1989)who found that malt extract, peptone and yeast extract as organic nitrogen sourses were the best nitrogen sources for the production of β-glucosidase by the yeast *Kluyveromyces fragilis*.

Table 9: Effect of differant nitrogen sources on the production of β-glucosidase by a shaken culture of *Rhodotorula glutinis*.

Nitrogen source	Growth O.D $_{600}$	β-glucosidase activity (Units/ml)
$NaNO_3$	2.1	130
KNO_3	1.5	110
NH_4NO_3	2.2	152
$Ca(NO_3)_2$	2.1	182
NH_4Cl	1.9	198
$(NH_4)\ H_2PO_4$	2.2	240
$(NH_4)_2HPO_3$	2.3	220
$(NH_4)_3PO_4$	2.1	190
$(NH_4)_2SO_4$	2.1	270

Incubation time 24h at 30°C.

Effect of NaCl concentration on the production of β-glucosidase by Rhodotorula glutinis:

To study the effect of NaCl on the production of β-glucosidase by marine yeast *Rhodotorula glutinis* , different concentrations were applied (0.2-1.0% w/v).The results in table(10) show that NaCl at a concentration of 0.6% was the best concentration for β-glucosidase production (280 units/ml).

Table 10: Effect of different concentration of NaCl on the production of β-glucosidase by a shaken culture of *Rhodotorula glutini*.

NaCl conc.% (w/v)	Growth O.D $_{600}$	β-glucosidase activity (Units/ml)
control	2.5	250
0.2	2.4	240
0.4	2.4	250
0.6	2.5	280
0.8	2.5	260
1.0	2.3	220

Incubation time 24h at 30°C.

Partial purification of β-glucosidase:

Rhodotorula glutinis β-glucosidase was precipitated by different concentratios of ammonium sulfate. The obtained results are givin in Table (11). The best recovery (75%)with the highest specific activity (4.14). A purification fold (3.02) was obtained at a concentration of 60% ammonium sulfate.

Table 11: Partial purification using ammonium sulphate precipitation.

Saturation %	Total protein(mg)	Total enzyme activity (units)	Specific activity (U mg^{-1})	Recovery %	Purification fold
Crude extract	350	480	1.37	100	1.0
20	30	5	0.17	1.04	0.12
40	45	21	0.47	4.38	0.34
50	47	45	0.96	9.38	0.70
60	87	360	4.14	75	3.02
70	57	25	0.44	5.21	0.32
80	45	21	0.47	4.38	0.34
90	53	13	0.25	2.71	0.18

Some properties of the partial purified enzyme:

1-Effect of pH-value:

Enzyme activity was assayed at different pHs ranging from pH 3.6 to 8.0. The result showed that β-glucosidase activity has an optimal pH at 6.5 (Fig.2). These results are agreement with Flemig and Duerksen(1967) on *Saccharomyses fragilis* Y-18, *Saccharomyses dobzhanskii* Y-19, Selim *et al.,* (1991) on *Kluyveromyces fragilis* β-glucosidase. While Saha *et al*., (1994) found that the optimum pH for β-glucosidase activity produced by *Aureobasidium pullulans* was at pH 4.5.

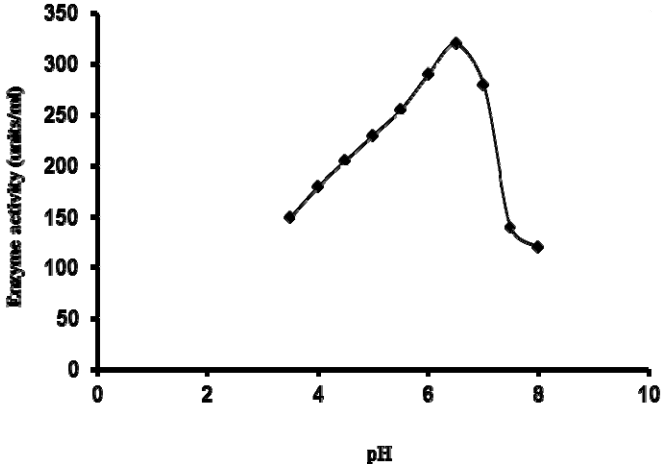


Fig. 2: Effect of pH on enzyme activity produced by *Rhodotorula glutinis.*

Effect of temperature on the activity and stability of partially purified enzyme:

The thermo activity and thermo stability of the partially purified β-glucosidase from *Rhodotorula glutinis* are shown in (Fig.3&4). The enzyme exhibited maximum activity at 45°C under the assay conditions used(Fig. 3). The partially purified enzyme was stable up to 30°C for 1h and it retained by about 50% at 55°C and 33.3% at 60°C(Fig.4). These results indicated that the enzyme was sensitive to heat above 45°C.

The results are agreement with that obtained by Selim *et al.*,(1991) with *Kluyveromyces fragilis* β-glucosidase and Korish and Salem(2007) with *Trichosporon* sp. β-glucosidase

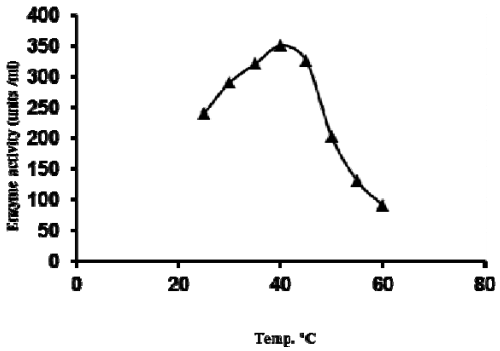


Fig. 3: Effect of temperature on enzyme activity produced by *Rhodotorula glutinis.*

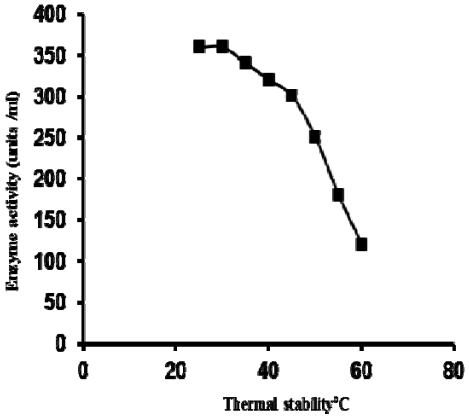


Fig. 4: Thermal stability of enzyme activity produced by *Rhodotorula glutinis.*

Substrates specificity of β-glucosidase:

The relative rates of hydrolysis of various substrates by partial purified β-glucosidase are presented in table (12). The enzyme hydrolyzed p-nitrophenyl-β-D-glucopyranoside effectively (100%) followed by cellobiose (65%) and maltose (43%). β-glucosidase had a little activity on p-nitrophenyl-α-D-glucopyranoside(≤7%) and p-nitrophenyl-β-D-galactopyranoside (≤ 3%).

Table12: Relative initial rates of hydrolysis of various substrates by partial purified β- glucosidase from *Rhodotorula glutinis*.

Substrate	Type of linkage	Relative hydrolysis rate %
p-nitrophenyl-β-D-glucopyranoside	βGlc	100
p-nitrophenyl-α-D-glucopyranoside	αGlc	7
Cellobiose	β-(1-4)Glc	65
Maltose	α-(1-4)Glc	43
p-nitrophenyl-β-D-galactopyranoside	β-(1-4)	3

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