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CHARACTERIZATION OF INVERTASE AND ALPHA AMYLASE FROM TWO FUNGAL SPECIES, PENICILLIUM *LILACINUM* AND *ASPERGILLUS NIGER*

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ABSTRACT

Amylases are one of the key enzymes that are used in food and other biotechnological applications. These enzymes hydrolyze starch into polymers composed of glucose units and have prospective application in food, fermentation and pharmaceutical industries. Although Amylases can be obtained from sources such as plants, animals and microorganisms, the enzymes from fungal and bacterial species show profound applications in industries. We have identified two fungal species, *Penicillium lilacinum* and *Aspergillus niger* that efficiently produce invertase and *α*-amylase. We have also identified optimal conditions for their production. The highest quantity of invertase (13.05 U/mL) was obtained from *Penicillium lilacinum* under these conditions: CM1, culture medium; yeast extract, nitrogen source; date syrup, carbon source; 96 h, incubation time period; culture medium pH 8.0; 40°C, incubation temperature 40°C; inoculum size conidia $6x10^6$, agitation rate 200 rev/min. The highest amount of *α*-amylase (8.14 U/mL) was obtained by *Aspergillus niger* under conditions: M1, culture medium; yeast extract as, nitrogen source; molasses as carbon source; incubation time period 72 h; initial pH of culture medium 6.5; incubation temperature 40°C; inoculum size $5x10^6$ conidia; agitation rate150 rev/min. These strains are potential candidates for industrial use because these enzymes maintain their activities even at harsh pH and temperature conditions.

Keywords: *α*-amylase, Commercial enzyme, Industrial enzyme, Invertase, Submerged fermentation.

INTRODUCTION

Alpha-amylase or 1, 4-alpha-D-glucan glucanohydrolase, E. C. 3.2.1.1, is an extracellular enzyme, which splits α-1, 4- glycosidic bonds of starch (a polysaccharide and composed of amylopectin and amylose) and produces alpha limit dextrin, maltose and glucose (Ahmed *et al*., 2015b; 2015c). Invertase and amylase have been extensively used commercial enzymes in medicinal, clinical, food and analytical chemistry and also in the brewing, baking, paper, pharmaceutical, detergent and textile industries (Ahmed *et al*., 2015b; 2015c; Sundarram and Murthy, 2014).

In many countries agricultural wastes are usually burnt in open air that can add to air pollution. By using agricultural (Cellulosic) wastes as energy source at least two targets can be achieved; on one hand air pollution can be contained and at the same time useful products can be produced. A body of literatures has reported nonconventional energy sources such as cassava starch, date syrup, starch, cotton stalk, rice husk, wheat straw, potato peel, sunflower waste, oilcakes, tapioca, fruit peel, corn and many others have been used in the fermentation process for enzymes production (Ahmed *et al*., 2015a, 2015b).

At the moment the biggest commercial application of amylases are in food industries. The biochemical multiplicity of microorganisms makes them reasonable sources of a varied selection of enzymes for use in food and other biotechnological usage (Mamma *et al*., 2008; Ahmed *et al*., 2015b; 2015c; Kulshrestha, 2013; Ahmed *et al*., 2015b; 2015c). We have investigated fungal α-amylases from *Penicillium lilacinum* and *Aspergillus niger*, physical and chemical constraint, and the use of these enzymes in industrial and other applications. These fungal species were grown on various agricultural based cellulosic wastes.

MATERIALS AND METHODS

Optimization of Enzyme Production Parameters:

All experiments were done in such a way that the parameter optimized in one experiment was fixed in the subsequent experiments for the maximum production of enzyme. Following were parameters:

Culture media: First of all the most suitable culture medium was determined. For optimization of *α*-Amylase production following culture media were used having composition (g/L).

M1: Dextrose 10, Peptone 5, Epsom salt 5, KH_2PO_4 5, Common salt 2.5, ferrous sulphate hepta hydrate .01, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydrochloride 0.001 (Burrel *et al.,* 1966).

M2: Soluble starch 20, $NH_4NO_3$10, KH_2PO_4, 14, KCl, 0.5, Epsom salt 0.1, $FeSO_4.7H_2O$, 0.01 (Matthias, 2013).

M3: NaCl 0.8, KCl 0.8, $CaCl_2$ 0.1, Na_2HPO_4 2.0, $MgSO_4$ 0.2, $FeSO_4$ 0.1, 8.0 Glucose, NH_4Cl 2.0 (Khan and Yadav, 2011).
M4: $ZnSO_4.7H_2O$ 0.062, $FeSO_4$ 0.068, copper sulphate pent hydrate 0.0001 and wheat bran 100 (Hayashida and Teramoto, 1986).
For invertase following were composition of culture media
CM1: Dextrose 10, peptone 5, Epsom salt 5, KH_2 PO_4 5, common salt 2.5, ferrous sulphate hepta hydrate 0.01, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydrochloride 0.001 (Burrel *et al*.,1966).
CM2: Yeast extract 10, peptone 20 and sucrose 20 (Dworschock and Wickerham, 1961).
CM3: Yeast extract 20, peptone 40, sucrose 20, $KH_2(PO_4)_2$ and Epsom salt 1 (Souza *et al*., 2007).
CM4: $NaNO_3$ 3, KCl 0.5, Epsom salt 0.5, ferrous sulphate hepta hydrate 0.01, K_2HPO_4 1, Sucrose 30 (Almeida *et al*., 2005).
CM5: Sucrose 40, corn steep liquor 30, $NaNO_3$ 3, KH_2PO_4 0.5, Epsom salt 0.05, $CaCO_3$ 2.5 (Poonawalla *et al*., 1965).

After the selection of the most suitable culture medium carbon source, nitrogen source, initial pH of culture medium, incubation temperature, conidia count and agitation rate were checked for the maximum production of enzymes in a sequence (Ahmed *et al*., 2015a, 2015b)

Invertase activity

Invertase activity was determined as described by Ahmed *et al.* (2015a; 2016), In brief, 0.1 mL of enzyme sample was mixed with 2.5 mL acetate buffer (50 mM, pH 5.5) and 0.1 mL 300 mM of sucrose; then incubated for 5 minutes at 35^o C, then added 1.0 mL of dinitro Salicylic acid. All contents then boiled for five minutes and noted Absorbance at 540 nm.

***α*-Amylase activity**

α- Amylase Activity was determined as described by Ahmed *et al.* (2015b; 2015c). In brief, 1.0 mL of enzyme sample is mixed with 1.0 mL of soluble starch (1 % w/v) in 50 mM sodium phosphate buffer at pH 7.0 and then incubated for 5 min at 50^o C, then added 1.0 mL dinitro Salicylic acid and then boiled for five minutes and noted Absorbance at 540 nm.

Table 1. Optimal Conditions for the production of invertase by various fungi.

Fungi	Culture medium	Incubation time period	Carbon source	Nitrogen source	Incubation Temperature	Initial pH	Inoculum Size	Agitation Rate	Optimized invertase activity
		h			^{o}C		Conidia/mL	Rev/min	U/mL
A. niger	CM1	72	Molasses	yeast extract	40	6.0	$5x10^6$	150	8.23
A. fumigatus	CM1	48	Sunflower waste	yeast extract	30	6.5	$6x10^6$	100	6.37
P. notatum	CM1	48	Molasses	yeast extract	40	6.5	$6x10^6$	200	6.41
P. lilacinum	CM1	96	date syrup	yeast extract	40	8.0	$6x10^6$	200	13.05
M. geophillus	CM1	48	molasses	yeast extract	35	6.5	$5x10^6$	150	5.25
P. expansum	CM1	48	date syrup	yeast extract	35	5.0	$6x10^6$	150	6.37

RESULTS

Determine optimal conditions for invertase production

We obtained the maximum quantity of invertase (13.05 U/mL) was obtained by *Penicillium lilacinum* (Table 1) under conditions: CM1, culture medium; yeast extract as nitrogen source; date syrup as, carbon source ; 96 h of

incubation time period; 8, initial pH of culture medium; 40° C, incubation temperature; $6x10^6$ conidia, inoculum size; 200 rev/min, agitation rate. Optimal conditions for the maximum production of invertase by other fungi were *Aspergillus fumigatuus,* 8.23 U/mL; *Aspergillus niger,* 6.24 U/mL; *Penicillium notatum,* 6.41 U/mL; *Penicillium lilacinum,* 13.05 U/mL; *Mucor geophillus,* 5.25 U/mL and *Penicillium expansum,* 6.37 U/mL) are mentioned in Table 1.

Determine optimal conditions for *α*-amylase production

Describe in sentence-Maximum amount of *α*-amylase (8.14 U/mL) by *Aspergillus niger* (Table 2) was produced under conditions: : M1, culture medium; yeast extract , nitrogen source molasses (how much), carbon source; 72 h, incubation time period; 6.5, initial pH of culture medium; 40° C, incubation temperature; $5x10^6$ conidia, inoculum size; 150 rev/min, agitation rate. Maximum amount of *α*-amylase by other fungi (*Aspergillus fumigatu,* 7.01 U/mL, *Penicillium notatum,* 6.58 U/mL; *Penicillium lilacinum,* 7.68 U/mL; optimal conditions for the production of *α*-amylase by *Mucor geophillus,* 4.87 U/mL and *Penicillium expansum,* 5.62 U/mL) are mentioned in Table 2.

Table 2. Optimal conditions for the production of alpha amylase by various fungi

Fungi	Culture medium	Incubation time period	Carbon source	Nitrogen source	Incubation Temperature	Initial pH of Medium	Inoculum Size	Agitation Rate	Optimized a-amylase activity
		h			^oC		Conidia/mL	Rev/min	U/mL
A. niger	M1	72	molasses	yeast extract	40	6.5	$5x10^6$	150	8.14
A. fumigatus	M1	72	Sunflower waste	casein	35	5.5	$6x10^6$	150	7.01
P. notatum	M1	48	molasses	corn steep liquor	30	5.5	$5x10^6$	150	6.58
P. lilacinum	M1	96	molasses	yeast extract	40	7.5	$6x10^6$	200	7.68
M. geophillus	M1	48	molasses	yeast extract	35	6.5	$5x10^6$	150	4.87
P. expansum	M1	72	molasses	yeast extract	35	5.5	$5x10^6$	150	5.62

DISCUSSION

The production of enzyme depends upon various parameters such as carbon source, nitrogen source, the strain, culture medium, incubation time period, incubation temperature, initial pH of culture medium, agitation rate and inoculums (Ahmed *et al*., 2015a; 2015b; 2015c, 2016).. Uma *et al.* (2010) reported Fruit peel as the most appropriate carbon source for the production of invertase by *Aspergillus flavus,* Pomegranate peel by Uma *et al*. (2012) and Sugar cane bagasse by Guimarães *et al.*, (2007). While for *α*- amylase production, Pomegranate peel (Singh *et al*., 2014), Wheat bran (Khan and Yadav, 2011), and starch ((Saleem and Ebrahim, 2014) were reported as the most appropriate carbon source.

Nitrogen source is also important constituent of Culture medium. Yeast extract (Uma et al., 2010), Yeast extract plus peptone (Olusanya and Oliotula, 1994), Peptone (Belcarz *et al*., 2000) and Corn steep liquor (Chan *et al*., 1991) have been reported as the most suitable nitrogen source for the maximum production of invertase while for *α*-amylase, Beef extract (Singh *et al*., 2014), Peptone (Khan and Yadav, 2011), Ammonium nitrate (Matthias, 2013) and Peptone and ammonium sulphate (Saleem and Ebrahim, 2014) have been reported.

Incubation time period is also important parameter for enzyme production. In literature 96 h (Uma *et al.*, 2010),

24 h (Zafar and Aslam, 2013) and 48 h (Mizunaga *et al*., 1981) are reported as the best incubation time period for the production of invertase while for *α*- amylase, 144 h (Singh *et al*., 2014), 48 h (Khan and Yadav, 2011) and 120 h (Matthias, 2013) have been reported.

Initial pH of culture medium is important for quality and quantity of culture medium. In order to obtain maximum quantity of invertase, initial pH of 5.0 (Uma *et al.*, 2010), 4.0 (Uma *et al.*, 2012) and 8.0 (Qureshi *et al*., 2012) were reported as the best while for *α*- amylase, a pH of 6.0 (Singh *et al*., 2014), 6.2 ((Khan and Yadav, 2011), and 4.0 (Matthias, 2013) have been reported.

Growth of microorganism is influenced by incubation temperature. It has been reported that 30º C (Uma *et al.*, 2010) and 45º C (Qureshi *et al*., 2012) is the most suitable incubation temperature while for *α*- amylase production, 35º C (Singh *et al*., 2014), 28 º C (Khan and Yadav, 2011), 30º C (Saleem and Ebrahim, 2014) and 45º C (Matthias, 2013) have been reported.

CONCLUSION

The highest quantity of invertase (13.05 U/mL) was obtained by *Penicillium lilacinum* under conditions: CM1, culture medium; yeast extract, nitrogen source; date syrup, carbon source; 96 h, incubation time period; 8, initial pH of culture medium; 40° C, incubation temperature; $6x10^6$ conidia, inoculum size; 200 rev/min, agitation rate. While the highest amount of *α*-amylase (8.14 U/mL) was obtained by *Aspergillus niger* under conditions: : M1, culture medium; yeast extract, nitrogen source; molasses, carbon source; 72 h, incubation time period; 6.5, initial pH of culture medium; 40° C, incubation temperature; $5x10^6$ conidia, inoculum size; 150 rev/min, agitation rate.

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