OPTIMAL CONDITIONS FOR THE PRODUCTION OF INDUSTRIAL ENZYMES BY ASPERGILLUS NIGER USING AGRICULTURAL WASTES AS SOURCE OF CARBON

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Abstract

Optimal conditions of cultural medium in submerged fermentation were studied for the production of industrial enzymes Invertase, α-Amylase and Glucose isomerase from *Aspergillus niger* using agricultural wastes (sunflower waste, cotton stalk and rice husk from fields of Interior Sindh) as sources of carbon. Effects of incubation time period (24-240 hours), source of nitrogen (Corn steep, Casein Peptone Potassium Nitrate Albumin A. Sulphate Urea Yeast Extract), pH (4-9) and temperatures (40-60°C) were also investigated for optimum enzymes production. It was found that the strain produced the highest level of Invertase (6.92 U/mL) in culture medium containing yeast extract as a source of nitrogen; 0.6*N* H_2SO_4 hydrolysed sunflower waste as a source of carbon after 96h of incubation at initial pH 8 and temperature 40°C. pH and thermo stable strain can be used for Invertase production in industries. Although α-amylase and glucose isomerase were also produced by the strain and even their activities (α-Amylase 0.78 U/mL; Glucose isomerase 0.69 U/mL) were enhanced by nitrogen sources but activities were too low for the utilization in industries.

Introduction

α-Amylase (Enzyme Code. 3.2.1.1) is industrially important enzyme covering around 30 % of total enzyme market in the world (Maarel *et al.,* 2002; Gupta *et al.,* 2003; Aiyer, 2005; Singh & Gupta, 2014). Previously, amylases were used in baking and ethanol fermentations. With the advent of biotechnology in past decades, amylases especially from microbial sources have found wide applications in various industries, mainly food, pharmaceutical, detergent etc. (Gupta *et al.,* 2003; Singh & Gupta, 2014).

Glucose isomerase (Enzyme Code.5.3.1.5) is one of the highest valued enzymes, amylases and proteases being the other two (Bhosale *et al.,* 1996; Sukumar *et al.,* 2013). This enzyme glucose to fructose (Chen, 1980; Sukumar *et al.* 2013). A corn syrup having high concentration of fructose is produced by glucose isomerase (Lee & Zeikus,1991; Sukumar *et al.,* 2013).

Invertase (Enzyme Code .3.2.1.26), splits sucrose into glucose and fructose. (Kulshrestha. *et al.,* 2013). It is one of the most widely used industrial enzymes, which is used to obtain invert sugar syrup from cane sugar (Ahmed, *et al.,* 2011). Specific interest has been focused on agriculture waste like sunflower cotton stalk rice husk which were obtained from the fields of Interior Sindh (Sanghar and Khairpur Districts) because they are usually disposed of by open burning causing pollution. Agricultural wastes have potential for conversion into useful products (Mamma *et al.,* 2008). In the present work the secretion of α-amylase, glucose isomerase and invertase by *Aspergillus niger* in submerged fermentation is being reported.

Materials and Methods

Strain: *Aspergillus niger* was obtained from the Institute of Biotechnology & Genetic Engineering University of Sindh and the culture was maintained following Dahot, (1986).

Culture Medium: Culture medium was prepared as reported by Burrel *et al.,* (1966). Composition was (g/L) Dextrose 10, Peptone 5, Epsom salt 5, KH_2PO_4 5, Common salt 2.5 and Ferrous sulphate hepta hydrate 0.01. Dextose of the medium was replaced by hydrolysed agricultural wastes.

Preparation of Spore Suspension: sterilized water 10 mL was added to the culture and then surface was rubbed to form suspnsion (Dahot & Memon, 1987). One mL of spore suspension contained around 5.8×10^7 conidia/mL which was determined by haemocytometer.

Hydrolysis of Agriculture Waste: Agricultural wastes were grinded to convert it into powdered form. Ten gram each of these was hydrolysed as described by Dahot and Abro (1994).

Cultivation Conditions: Culture broth of 100 mL was added with agriculture waste soluble filtrate in a conical flask and autoclaved at 1.5 kg/cm^2 for 20 min. as described by Dahot and Memon (1987).

Determination of Enzyme Activity: Activities of amylase and invertase was determined by Bernfeld method (1955). One unit of invertase activity is the amount of enzyme which releases 1 µg of reducing sugar at 37^{o}C per minute while one unit of amylase is the amount of enzyme which releases 1 µmol of reducing sugar as maltose per minute. Activity of glucose isomerase was determined by the method of Dische and Borenfreund (1951).one unit of glucose isomerase is the amount of enzyme which is required to convert 1 µmol of glucose to fructose in one minute at pH 7, temperature 60^{o}C and 300g/L glucose concentration.

Results and Discussion

A number of nonconventional carbon sources such as starch, oilcakes, cassava starch, potato, corn and tapioca have been used in submerged fermentation for various enzymes production (Krishnan & Chandra, 1982; Sani *et al.*, 1992; Negi & Benerjee, 2006; Sivaramakrishnan *et al.*, 2006; Mahdi *et al.*, 2012). In the present work we have used sunflower waste cotton stalk and rice husk which are agricultural wastes and in our country they are usually disposed of by open burning which causes pollution. There may be two objectives which can be achieved by using agricultural wastes as sources of carbon. On one hand they can be used as raw materials for the production of valuable enzymes and other useful products while on the other hand pollution problem can be reduced. All sources of carbon were hydrolyzed by 0.3 and 0.6 *N* H_2SO_4.The effects of hydrolysed agricultural waste on the production of α-amylase, glucose isomerase and invertase by *Aspergillus niger* were studied and results are presented in Table 1, 2, and 3. It is clear from these tables that incubation time period and optimum quantity of enzymes vary with carbon source and higher values are observed when 0.6 *N* H_2SO_4 hydrolysed sunflower waste (Table 1) cotton stalk (Table 2) and rice husk (Table 3) were used as a source of carbon while lower values in case of 0.3*N* H_2SO_4 hydrolysed agricultural wastes (Table 1, 2, and 3). All enzymes showed maximum activity around 96hours of incubation time but on continued incubation decreased which may be due to denaturing of enzyme or synthesis of some other inhibiting metabolites. (Kulshrestha. *et al.*, 2013). These results are discussed below for individual enzyme.

Table 1. Effect of incubation time and different concentrations of H_2SO_4 on sunflower waste at 27° C and initial pH of 6.0

Conc.	Time period (h)	pH	Enzymes activities		
			Invertase (U/mL)	*α*-amylase (U/mL)	Glucose isomerase (U/mL)
0.3*N* H_2SO_4	24	4.37	1.52	0.34	0.29
	48	4.21	1.62	0.44	0.37
	72	5.01	1.74	0.53	0.43
	96	5.23	1.61	0.59	0.47
	120	5.42	1.52	0.48	0.41
	144	5.71	0.89	0.39	0.38
	168	5.79	0.77	0.32	0.32
	192	5.87	0.35	0.21	0.27
	216	5.99	0.24	0.18	0.19
	240	6.09	0.11	0.09	0.11
0.6*N* H_2SO_4	24	6.02	1.54	0.38	0.31
	48	5.75	1.67	0.46	0.39
	72	5.63	2.21	0.51	0.45
	96	5.73	2.78	0.64	0.51
	120	5.83	2.02	0.53	0.45
	144	5.98	1.48	0.47	0.38
	168	6.15	1.39	0.39	0.28
	192	6.23	0.67	0.28	0.21
	216	6.29	0.29	0.17	0.16
	240	6.28	0.17	0.07	0.13

Table 2. Effect of incubation time and different concentrations of H_2SO_4 on cotton stalk waste at 27° C and initial pH of 6.0.

Conc.	Time period (h)	pH	Enzymes activities		
			Invertase (U/mL)	*α*-amylase (U/mL)	Glucose isomerase (U/mL)
0.3*N* H_2SO_4	24	4.37	1.32	0.17	0.20
	48	4.21	1.32	0.24	0.27
	72	5.01	1.54	0.34	0.33
	96	5.23	1.41	0.35	0.37
	120	5.42	1.32	0.28	0.41
	144	5.71	0.69	0.21	0.28
	168	5.79	0.47	0.15	0.22
	192	5.87	0.35	0.12	0.17
	216	5.99	0.44	0.09	0.09
	240	6.09	0.01	0.01	0.01
0.6*N* H_2SO_4	24	6.02	1.34	0.28	0.24
	48	5.75	1.57	0.36	0.33
	72	5.63	2.11	0.38	0.36
	96	5.73	2.33	0.62	0.41
	120	5.83	2.11	0.35	0.30
	144	5.98	1.32	0.38	0.29
	168	6.15	1.21	0.24	0.21
	192	6.23	0.54	0.21	0.18
	216	6.29	0.21	0.16	0.11
	240	6.28	0.12	0.02	0.10

Table 3. Effect of incubation time and different concentrations of H_2SO_4 on rice husk waste at 27° C and initial pH of 6.0.

Conc.	Time period (h)	pH	Enzymes activities		
			Invertase (U/mL)	*α*-amylase (U/mL)	Glucose isomerase (U/mL)
0.3*N* H_2SO_4	24	4.37	1.19	0.13	0.23
	48	4.21	1.09	0.24	0.31
	72	5.01	1.21	0.29	0.36
	96	5.23	1.25	0.38	0.39
	120	5.42	1.11	0.29	0.51
	144	5.71	0.66	0.20	0.35
	168	5.79	0.54	0.19	0.24
	192	5.87	0.34	0.15	0.19
	216	5.99	0.12	0.09	0.14
	240	6.09	0.06	0.03	0.12
0.6*N* H_2SO_4	24	6.02	1.26	0.18	0.36
	48	5.75	1.41	0.31	0.39
	72	5.63	2.24	0.40	0.42
	96	5.73	2.39	0.59	0.49
	120	5.83	2.24	0.57	0.38
	144	5.98	1.26	0.34	0.31
	168	6.15	1.20	0.28	0.25
	192	6.23	0.67	0.22	0.23
	216	6.29	0.33	0.18	0.16
	240	6.28	0.24	0.09	0.14

Table 4. Enzymes activities (U/mL) of different nitrogen sources with 0.6*N* H_2SO_4 and their effect on different amounts (g) of hydrolysed sunflower waste.

S. No.	Name	0.25 g			0.50 g		
		Invertase	Alpha amylase	Glucose isomerase	Invertase	*α*-amylase	Glucose isomerase
1.	Corn steep	2.67	0.67	0.52	2.98	0.69	0.55
2.	Casein	2.78	0.61	0.54	3.01	0.64	0.58
3.	Peptone(control)	2.42	0.69	0.56	3.72	0.71	0.59
4.	Potassium Nitrate	2.34	0.68	0.54	3.18	0.70	0.58
5.	Albumin	2.79	0.62	0.56	3.52	0.65	0.57
6.	A. Sulphate	2.92	0.67	0.54	3.79	0.69	0.57
7.	Urea	3.07	0.71	0.53	4.01	0.76	0.61
8.	Yeast Extract	4.32	0.67	0.59	4.98	0.68	0.64

Table 5. Effect of pH with 0.6*N* H_2SO_4 hydrolysed sunflower waste.

S. No.	pH scale	Enzymes activities (U/mL)		
		Invertase	*α*-amylase	Glucose isomerase
1.	4.0	4.21	0.65	0.56
2.	4.5	4.72	0.66	0.57
3.	5.0	4.67	0.67	0.57
4.	5.5	4.92	0.66	0.58
5.	6.0	5.02	0.67	0.60
6.	6.5	5.21	0.69	0.60
7.	7.0	5.34	0.68	0.64
8.	7.5	5.67	0.71	0.68
9.	8.0	5.99	0.74	0.71
10.	8.5	4.98	0.70	0.67
11.	9.0	4.79	0.68	0.61

Table 6. Effect of temperature with 0.6*N* H_2SO_4 hydrolysed sunflower waste.

S. No.	Temperature (° C)	Enzymes activities (U/mL)		
		Invertase	*α*-amylase	Glucose isomerase
1.	20	5.79	0.69	0.65
2.	25	5.82	0.69	0.66
3.	30	5.97	0.71	0.68
4.	35	6.21	0.73	0.68
5.	40	6.92	0.78	0.68
6.	45	6.60	0.72	0.67
7.	50	6.21	0.67	0.65
8.	55	6.02	0.65	0.62
9.	60	6.01	0.61	0.60

***α*-amylase:** *α*-amylase production was maximum (0.64 U/mL)when *Aspergillus niger* was cultured at 0.6 *N of* H_2SO_4 hydrolysed sunflower waste as a source of carbon after 96 h in the submerged fermentation (Table 1).Enzyme activity was slightly enhanced (0.78 U/mL) around a temperature of 40^{o}C (Table 6) and at initial pH around 8.0(0.74 U/mL in Table 5).When cotton stalk (Table 2) and rice husk (Table 3) were used as sources of carbon, values were slightly lower (0.62 U/mL cotton stalk;0.59 U/mL rice husk) as compared to the sunflower waste (0.64 U/mL in Table 1). These results were agreed with other organisms but in case of *Aspergillus niger* enzyme activity was lower when compared with others. (Viswanthan & Surlikar, 2001; Ramachandran *et al.*, 2004; Singhania *et al.*, 2009; Bhardwaj *et al.*, 2011; Singh & Gupta, 2014). It showed that *α*-amylase production

for industrial purpose may not be achieved by *Aspergillus niger* although enzyme activity was enhanced by various nitrogen sources especially with urea (0.71 U/mL in Table 4).Interestingly the strain showed pH stability between 4-9 (Table 5) and thermo stability up to 60^{o} C (Table 6) which is a basic requirement for industrial use (Diomi *et al.,* 2008).

Glucose isomerase: Glucose isomerase production was maximum (0.49U/mL) when *Aspergillus niger* was cultured at 0.6 *N of* H_2SO_4 hydrolysed sunflower waste as a source of carbon after 96 h in the submerged fermentation (Table 1) Enzyme activity was slightly improved (0.75 U/mL) around a temperature of 40^{o} C(Table 6) and at initial pH around 8.0 (0.71 U/mL in Table 5). When cotton stalk (Table 2) and rice husk (Table 3) were used as sources of carbon then values were slightly lower (0.41 U/mL cotton stalk; 0.49 U/mL rice husk) as compared to the sunflower waste (0.51 U/mL in Table 1). The results were in agreement with other organisms but in case of *Aspergillus niger* enzyme activity was lower when compared with others. (Khire *et al.*, 1990; Lee & Zeikus,1991; Bhosale *et al.*, 1996; Lama *et al.*, 2001; Sukumar *et al.*, 2013). It showed that glucose isomerase production for industrial purpose may not be achieved by *Aspergillus niger* although enzyme activity was improved by various nitrogen sources especially with yeast extract (0.64 U/mL in Table 4). Interestingly the strain showed pH stability between 4-9 (Table 5) and thermo stability up to 60^{o} C (Table 6) which is a basic requirement for industrial use (Diomi *et al.*, 2008).

Invertase: Invertase production was maximum (2.78 U/mL) when *Aspergillus niger* was cultured at 0.6 *N* of H_2SO_4 hydrolysed sunflower waste as a source of carbon after 96 h in the submerged fermentation (Table 1). When cotton stalk (Table 2) and rice husk (Table 3) are used as a source of carbon then values were slightly lower (2.33 U/mL cotton stalk; 2.39 U/mL rice husk) as compared to the sunflower waste (2.78 U/mL in Table 1). The results were comparable with other organisms and fairly high values of enzyme activities (6.92 U/mL in Table 6) showed its feasibility for commercial use after further research (Dahot, 1986; Egorov *et al.*, 2000; Guimarães *et al.*, 2007; Luis *et al.*, 2007; Uroš *et al.*, 2010; Kulshrestha *et al.*, 2013).

Sources of nitrogen are very important because they have specific response to metabolism. (Silveira *et al.*, 2000). Various nitrogen sources (Corn steep, Casein Peptone(control) Potassium Nitrate Albumin A. Sulphate Urea Yeast Extract) were used to increase the production of invertase and yeast extract was found to be the best (4.98 U/mL in Table 4). Increase in enzyme activity may be due to the reason that various other enzymes are also produced which may affect at the production of each other (Egorov *et al.*, 2000).

A range of pH (4.0 to 9.0) was studied and found that pH of 8.0 would be the best for optimum enzyme production (4.98 U/mL in Table 5). However, various researchers have reported optimal pHs from 2.6 to 6.5 for invertase from different yeast and fungi (Quiroga *et al.*, 1995; Chaudhuri & Maheshwari 1996; Hocine *et al.*, 2000; Rubio *et al.*, 2002; Bhatti *et al.*, 2006).

Temperature of 40° C was optimum temperature (6.92 U/mL in Table 6) for the Invertase production, which was lower than that determined for other fungi (Dahot, 1986; Ashokumar *et al.*, 2001; Herwig *et al.*, 2001; Rubio *et al.*, 2002; Guimarães *et al.*, 2007; Luis *et al.*, 2007; Diomi *et al.*, 2008; Uroš *et al.*, 2010). Interestingly the strain showed pH stability between 4-9 (Table 5) and thermo stability up to 60^{o} C (Table 6) which is a basic requirement for industrial use (Diomi *et al.*, 2008).

Conclusion

In the present study local fungus *Aspergillus niger* was used to produce invertase amylase and glucose isomerase by using agricultural wastes as sources of carbon. The strain was pH and thermo stable therefore can be used in industries for enzymes productions. Higher Invertase activity(6.92 U/mL) showed that *Aspergillus niger* is fit for industrial use for the invertase production but lower α-Amylase(0.78 U/mL) and Glucose isomerase (0.69 U/mL) activities showed that they may further be investigated for commercial use.

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