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Biosynthesis of invertase by *Penicillium chrysogenum* using solid state fermentation technique

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Abstract

Invertases are the enzymes which hydrolyze sucrose into D-glucose and D- fructose. The hydrolyzed products are the commercial carbohydrates. Invertases are of great industrial importance due to its applications in the cosmetics, feeding of the animals, detoxifying process of cassava, modification of natural polymers, synthesizing the organic chemicals, for decolorization of the ink from the paper to be recycled and the process for the wet grain milling**.** The present study is concerned with production of invertase from locally isolated *Penicillium chrysogenum*. The maximum production of invertase (11.97±0.03 U/ml/min) was obtained at incubation temperature of 50°C growing on 10g of wheat bran after 72h of incubation when 1m of spore inoculum was used at pH 5 and 10 ml of the acetate buffer as moistening agent.

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Introduction

Invertases are produced by several organisms like plants (Pressey, 1966; Kim, 1980; Obenland *et al*., 1993;) and micro-organisms (Abrams *et al*., 1994; Zouaoui *et al*., 2016). The Invertase produced by *Penicillium chrysogenum* is found as the exo-enzyme (Damle *et al*., 1958). Fungal enzymes are important in food industry, detergent industry e.g. dry meat products and the enzymes produced by *Penicillium sp*. is well known in flavoring cheese (Benito *et al*., 2006). Invertases are the enzymes which hydrolyze sucrose into D-glucose and D- fructose (Sanchez *et al*., 2001; Alegre *et al*., 2009). Invertases belongs to the GH32 family of glucoside hydrolases (Alberto *et al*., 2004). The hydrolyzed products are the commercial carbohydrates (Strum, 2001). Invertase production by *Penicillium chrysogenum* has been studied widely (Neuro and Reyes, 2002). Transfructosylation is also done by invertases resulting in the formation of several isomers of ketoses of tri-saccharides (Obenland *et al*., 1993). Invertases are glycoprotein with residual material of mannose which is supposed to be component of carbohydrate sugar (Gascon *et al*., 1968; Chen *et al*., 1999). Invert sugar and the syrup i.e. high fructose syrup (HFS) and the fructose are preferably used in preparing the candies and jams (Aranda *et al*., 2006). Invertases are of great industrial importance due to its applications in the cosmetics, feeding of the animals, detoxifying process of cassava, modification of natural polymers, synthesizing the organic chemicals, for decolorization of the ink from the paper to be recycled and the process for the wet grain milling (Leitao *et al*., 2009). Pakistan has to import Invertase from other countries to fulfill its commercial needs and a large amount of money is spent for this purpose. Although researchers have made progress in this field but still the Invertase production has not increased to the level as required in Pakistan. In this work, we have produced Invertase from locally isolated *Penicillium* sp which will open new ways of progressing fermentation in biotechnological industries.

Materials and methods

The prepared pure cultures of the *Penicillium chrysogenum* were obtained from lab of Biotechnology and Mycology of the Department of Botany of Government College University Faisalabad. The fungus was preserved on Potato Dextrose Agar (PDA) slants at 4°C. The cultures were refreshed after every 15 days. The fungal inoculums were prepared by the addition of 10ml of distilled water into the cultured slants and then spores were scratched with the help of the needle and the spore inoculums thus obtained was used as inoculum.

Fermentation technique

The solid state fermentation technique was used in this work. The substrates used were Wheat bran, Rice bran, Soya bran, Corn bran and Baggase.10g of the substrate was added into the 250ml Erlenmeyer flasks. That basal substrate was then moistened by 10ml of distilled water and then shaken. Flasks were cotton plugged and sterilized at about 121°C and 15 bars pressure for 15 minutes.

Invertase assay

The activity of the invertases was determined after the method of Sumner and Howells (1935) with little modifications. About 0.1ml of the enzyme extract was taken in a test tube with the 0.9 ml of the acetate buffer of pH 5 and molarity 0.03M. 1ml of the DNS was added to the above solution in order to stop the reaction followed by heating for about 5 minutes. The absorbance was measured by spectrophotometer and the wavelength was adjusted at 540nm. One unit of the Invertase (IU) is defined as the amount of enzyme which releases 1µ moles of the glucose/minute/ml which was under the described conditions.

$$U\backslash ml\backslash min = \frac{\text{Sugar released}/2 \times 100}{180 \times 60}$$

The protein in enzyme was measured through Bradford method (Bradford, 1976) using Bovine serum Albumin solution (BSA) as standard.

Statistical analysis

The data collected were subjected to analysis of variance techniques (ANOVA) by using Costat software cs6204W, where the difference means was significant ($p \leq 0.05$), the mean values were compared using least significant difference (LSD).

Results

Effect of incubation temperature

Different incubation temperatures were selected for the productivity of the Invertase i.e. 20°C, 30°C, 40°C, 50°C, 60°C and the 70°C (Table 1).

Table 1. Effect of incubation temperature on production of Invertases by *Penicillium chrysogenum* through solid state fermentation technique.

SL	Incubation temperature (°C)	Invertases Activity (U/mL/min)	Proteins (mg/mL)	Specific activity (U/mg)
1	20	7.63± 0.26^{b}	1.47±0.07^{b}	5.19
2	30	5.93± 0.84^{c}	0.35±0.08^{e}	16.94
3	40	3.77± 0.45^{d}	1.74±0.1^{a}	2.167
4	50	9.45± 0.65^{a}	0.625±0.03^{d}	15.12
5	60	1.22±0.01^{f}	0.49±0.42^{de}	2.49
6	70	2.21± 0.01^{e}	0.93±0.02^{c}	2.38
	LSD	0.8672	0.1616	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance is 5%.

Maximum production (9.45± 0.65 U/mL/min) was obtained on the incubation temperature of 50°C while minimum (1.22±0.01U/mL/min) production observed at 60°C.

Selection of different substrates

For the optimization of the productivity of *Penicillium chrysogenum* different substrates were used for the activity of the Invertases (Table 2).

Table 2. Selection of substrate for production of Invertases by *Penicillium chrysogenum* through solid state fermentation.

SL	Substrates	Invertases activity (U/mL/min)	Proteins (mg/mL)	Specific activity (U/mg)
1	Wheat bran	9.49±0.26^{a}	0.61±0.01^{a}	15.56
2	Rice bran	6.14±0.27^{b}	0.52±0.15^{b}	11.80
3	Baggase	3.07±0.46^{d}	0.42±0.02^{c}	7.31
4	Corn bran	1.55±0.66^{e}	0.28±0.10^{d}	5.54
5	Soybean meal	3.35± 0.42^{c}	0.36±0.01	9.31
	LSD	0.8091	0.1558	

The substrates used were the wheat bran, rice bran, corn bran, Soya bran and the baggase. The minimum activity (1.55±0.66U/mL/min) of Invertases was observed when corn was used as substrate. The maximum activity (9.49±0.26 U/mg) was found on the wheat bran.

Effect of different quantities of substrates

Different quantities of the substrates were used for the productivity of the Invertases i.e., 5g, 10g, 15g, 20g, 25g, 30g and 35g respectively (Table 3).

The maximum production (9.97±0.42^{a} U/ml/min) of the Invertase was found on the 10g of the substrate while the minimum quantity (3.25±0.12^{f} U/ml/min) was on the 35g of substrate.

Effect of rate of fermentation

The incubation time was optimized in order to check the production of invertases (24h, 48h, 72h, 96h, 120h and the 144h). The production of the Invertase by the *Penicillium chrysogenum,* shown in Table 4 which depicts that the maximum productivity

(9.54±0.64 U/ml/min) of the Invertase was achieved after 72h of incubation. The minimum activity (0.31±0.05 U\ml\min) of the Invertase was obtained after 144h of fermentation.

Table 3. Effect of different quantities of substrates on production of Invertases by *Penicillium chrysogenum* through solid state fermentation technique.

SL	Quantity of substrates (g)	Invertases activity (U/mL/min)	Proteins (mg/mL)	Specific activity (U/mg)
1	5	2.14±0.44^{g}	0.40±0.08^{e}	5.35
2	10	9.97±0.42^{a}	0.56±0.03^{de}	17.80
3	15	8.14±0.40^{b}	0.78±0.18^{cd}	10.44
4	20	7.2±0.50b^{c}	0.89±0.15^{bc}	8.09
5	25	5.14±0.77^{d}	1.02±.004^{abc}	5.04
6	30	4.44±0.18^{e}	1.15±0.06^{ab}	3.86
7	35	3.25±0.12^{f}	1.29±0.27^{a}	2.52
	LSD	0.81	0.28	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance is chosen as 5%.

Effect of different inoculums size

The quantity of the inoculums used for the productivity of the Invertases was 0.5ml, 1ml, 1.5ml, 2ml, 2.5ml and 3ml respectively (Table 5). The maximum productivity (9.96±0.01 U/ml/min) of the enzyme was obtained with the inoculums size of about 1ml. The minimum activity (1.56±0.19U/ml/min) of Invertase was by adding inoculum of 3ml.

Table 4. Effect of rate of fermentation on production of Invertase by *Penicillium chrysogenum* through solid state fermentation.

SL	Time of incubation	Invertases activity (U\mL\min)	Proteins (mg\mL)	Specific activity (U\mg)
1	24	5.46±0.39^{c}	0.80±0.09^{d}	6.83
2	48	6.76±0.14^{b}	1.72±0.01^{b}	3.93
3	72	9.54±0.64^{a}	0.99±0.01^{c}	9.64
4	96	2.24±0.10^{d}	2.25±0.01^{a}	0.996
5	120	1.18±0.09^{e}	0.6±0.13^{e}	1.97
6	144	0.31±0.05^{f}	0.31±0.01^{f}	1.00
	LSD	0.6513	0.1617	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance is 5%.

Effect of varying initial pH of moistening agent

Different pH of the acetate buffer was selected for the productivity of the Invertases i.e., 3, 5, 7, 9, and 11 respectively (Table 6).

The maximum production (11.03±0.35 U/ml/min) was obtained on the pH 5 and minimum production (5.415±0.49 U/ml/min) was obtained at pH 11.

Effect of moistening level

Varied level of the moistening agents were used for the production of the Invertases i.e., 5ml, 10ml, 15ml, 20ml, 25ml 30ml etc (Table 7). Maximum production (11.39±0.36 U/ml/min) was at 10ml while it was found to be minimum (0.58±0.17 U/ml/min) at 25ml.

Effect of different Moistening agents

Different moistening agents were selected for the productivity of

the Invertases i.e., Acetate buffer, phosphate buffer, distilled water and the tap water (Table 8). The maximum production (11.97±0.03 U/ml/min) was observed on the acetate bufferwhile the minimum reading (5.02±0.16 U/ml/min) was obtained on the tap water.

Table 5. Effect of different inoculums sizes on production of Invertases by *Penicillium chrysogenum* through solid state fermentation technique.

SL	Inoculums size (mL)	Invertases activity (U/mL/min)	Proteins (mg/mL)	Specific activity (U/mg)
1	0.5	5.79± 0.39^{cd}	1.32±0.01^{d}	4.39
2	1	9.96±0.01^{a}	2.09± 0.01^{a}	4.77
3	1.5	8.47±0.01^{b}	1.55± 0.02^{c}	5.47
4	2.0	3.23±0.31^{f}	1.15±0.10^{e}	2.81
5	2.5	7.26±0.01^{bc}	1.73±0.08^{b}	4.20
6	3.0	1.56±0.19^{e}	0.98±0.14^{f}	1.59
	LSD	0.6251	0.2347	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance chosen 5%.

Discussion

For the present work *Penicillium chrysogenum* was used to check its potential for Invertase production. A strain of *Penicillium chrysogenum* was obtained from the laboratory of Mycology and Biotechnology, Department of Botany Government College University Faisalabad.

Effect of incubation temperature

The optimum temperature for production of Invertase was 50°C by experimental results. Further increase in incubation temperature decrease enzyme activity. Comparison with the published research papers showed that the results are inline with the findings of Uma *et al*., (2010), Alegre *et al*., (2009), Patil *et al*., (2010) and Shaheen *et al*., (2007). Chen and Liu, (1999) and Rustiguel, (2010) did not agree with the findings of the present work. At high incubation temperature the enzyme activity was not significant because of denaturing process at the active site of the enzyme (Russo *et al*. 1996).

Table 6. Effect of varying initial pH of moistening agent on Production of Invertases by *Penicillium chrysogenum* through solid state fermentation.

SL	pH	Invertases activity (U/mL/min)	Proteins (mg/mL)	Specific activity (U/mg)
1	3	8.205±0.48^{b}	0.38±0.07^{c}	21.59
2	5	11.03±0.35^{a}	0.22±0.03^{b}	50
3	7	7.575±0.63^{c}	0.5±0.01^{e}	15.14
4	9	6.575±0.70^{d}	0.3±0.04^{d}	21.91
5	11	5.415±0.49^{e}	0.61±0.01^{a}	8.87
	LSD	0.9608	0.0618	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance is 5%.

Effect of different substrates and their quantities

Among different agricultural byproducts checked wheat bran supported maximum yield of enzyme might be due to reason that it provides full nutrition for the growth of the organism and enzyme production and results are similar to Alegre *et al*. (2009). Patil *et al*. (2011) did not agree with the present findings. Different quantities of the selected substrate also plays important role towards enzyme production. Best yield was obtained with 10g of wheat bran. It might be due to the appropriate moisture contents and good aeration of the ideal quantity of substrate.

The present results are in accordance with the result of Shaheen *et al.* (2007) and Alegre *et al.* (2009). Invertase production by *Penicillium chrysogenum* was observed maximum after 72h incubation and is in line with work of Ashokkumar *et al.* (2001), Alegre *et al.* (2009), Rustiguel *et al.* (2010), Uma *et al.* (2010) and Giraldo *et al.* (2011).

Effect of different inoculum size and pH

The production of the enzyme was maximum with1ml of spore inoculums as reported by Shafiq *et al.* (2003), Haq *et al.* (2007) and Bokosa *et al.* (1992).

The highest productivity of the extra-cellular Invertase was achieved when pH was adjusted to 5. Further increase in pH decrease the extra cellular increase of enzyme. It might be due to the reason that basic pH stops the extra cellular enzyme production(Patil *et al.*, 2010) .The results are in line with the findings of previous work of Goosen *et al.*, (2007). This report showed contrary results with some of the previous workers Kaur and Sharma (2005).

Table 7. Effect of moistening level on the production of the Invertases by *Penicillium chrysogenum* through solid state fermentation technique.

SL	Type of moistening level (ml)	Invertases activity (U/mL/min)	Proteins (mg/mL)	specific activity
1	5	6.60±0.27^{b}	0.835±0.03^{c}	7.90
2	10	11.39±0.36^{a}	0.99±0.012^{b}	11.51
3	15	0.94±0.049^{e}	0.66±0.03^{d}	1.42
4	20	4.32±0.01^{d}	0.54±0.04^{e}	8.00
5	25	0.58±0.17^{f}	0.40±0.01^{f}	1.45
6	30	5.41±0.55^{c}	1.54±0.06^{a}	3.51
	LSD	0.81	0.11	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance is 5%.

Effect of different Moistening agents

Concentration of moistening agent plays important role in enzyme production. Among the various concentration 10ml of the distilled water as moistening agent was found optimum for enhanced Invertase production. It might be due to the reason that moistening of the substrate affects the porosity of the solid medium (Aranda *et al.*, 2006). Ten milliliter of the moistening agent is also reported by several workers (Gomez *et al.*, 2000).

Table 8. Effect of different moistening agents on production of Invertases by *Penicillium chrysogenum* through solid state fermentation technique.

SL	Types of moistening agents	Invertases activity (U/mL/min)	Proteins (mg/mL)	Specific activity (U/mg)
1	Acetate buffer	11.97±0.03^{a}	0.39±0.03^{c}	30.69
2	Tap water	5.02±0.16^{d}	0.25±0.05^{d}	20.08
3	Phosphate buffer	7.05±0.09^{c}	0.65±0.26^{b}	10.85
4	Distilled water	9.71±0.09^{b}	0.79±0.04^{a}	12.29
	LSD	0.27	0.36	

Each reading is the mean of triplicates.± shows standard deviation among these replications. Level of significance is 5%.

Type of the moistening agent also play important role in the production of Invertase from *Penicillium chrysogenum*. Among different moistening agents acetate buffer was optimized for high production of Invertase in contrast to distilled water as the optimized moistening agent was reported by Aranda *et al.*, (2006).

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