December 2009 Volume 3 Issue 4

Trade Science Inc.

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 3(4), 2009 [299-305]

A comparative study on invertase production from *Aspergillus fumigatus & Penicillium brevicompactum* by submerged fermentation

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Received: 3rd August, 2009 ; Accepted: 13th August, 2009

ABSTRACT

Biologically active enzymes may be extracted from any living organisms like plants, animals and microorganisms. Invertase splits sucrose into glucose and fructose (invert syrup) and can be applied for the production of HFCS which has application in food industries. Invertase is used to improve the shelf life of confectionaries. Invertase production by *Aspergillus spp.,* and *penicillium spp.,* at varying pH, temperature, sucrose concentration (carbon source), sugarcane bagasse (substituted carbon source) and yeast extract (as nitrogen source) concentration at period of incubation was studied by using Czapek Dox as basal medium. The results of the study revealed that the production of invertase by *Aspergillus spp.,* and *penicillium spp.,* was maximum on the 4th day of incubation at an optimum pH of 4.0 and 5.0 respectively. Optimum temperature is 30^{0}C for maximum production by both organisms. The maximum enzyme production was observed at 3 gm/ml (Sucrose) and 4 gm/ml (Sugar cane bagasse by using *Aspergillus spp.,* and *penicillium spp.,* and the concentration of the yeast extract (as nitrogen source) was 1.5gm/100ml for these organisms.

KEYWORDS

Invertase;
Aspergillus spp.;
Penicillium spp.;
Sugarcane bagasse.

INTRODUCTION

The majority of enzymes used in industrial/ biotechnological applications are derived from particular fungi and bacteria[1]. There are some biotechnological fields in which fungi play an important role, namely the production of food, extra cellular enzymes, and secondary metabolites and of organic chemicals. In each case fungi compete with other biological systems such as bacteria or cell cultures or with the chemical synthesis of the respective compounds.

Invertase production by microorganism such as *Aspergillus niger* ATCC 20611, *Zymomonas mobilis*[2] and *Clavibacter mechigan*[3] are known. *Penicillium purpurogenum* was used.[4] Thus, increasing potential of invertase application prompt screening for newer invertase producing microorganisms.

Invertases or β-D- fructofuranosidase are special kind of enzymes that catalyze the hydrolyses of sucrose. The enzyme cleaves α- 1-4 glycosidic linkage between α- D- glucose and β-D- fructose molecules of sucrose by hydrolysis producing monosaccharides such as glu-

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cose and fructose. β-D- fructofuranosidase are extracellular as well as intracellular enzymes.[5]

Sucrose is a natural sweetener, traditionally used in human nourishment due to the pleasant taste, nutritious value and low cost of production.[6] Hydrolyses of sucrose produces a fructose and glucose equimolar mixture named invert sugars. The inverted sugar is incorporated more easily in industrial preparations and has more added value than sucrose.[7] Different substrates can be used in submerged fermentation for the production of invertase. Sucrose is designated as the best sole carbon source for the production of the invertase.[8]

In this present study we report the capacity of production of thermo stable extracellular invertase produced by filamentous fungi *A.fumigatus* and *P.brevicompactum* using sugarcane bagasse as substrate. We have studied in detail the effects of various conditions contributing to the enhancement of extracellular invertase production by these organisms in submerged shaken cultures by using sugarcane bagasse.

MATERIALS AND METHODS

Isolation and enumeration of fungi from soil

A large number of fungi of different groups are found in soil. They constitute the major place among soil microorganisms .A small amount of soil sample was collected from four corners and centre of the sugarcane field by making a 'V' shaped pit. They were mixed to make one lot. 10g of the soil sample was weighed and then the sample was serially diluted with sterile distilled water. 1.0 ml of the diluted soil suspension was transferred aseptically into the PDA Agar plates [Supplemented with aureomycin antibiotic each 30 mg /L]. Gently rotate the plate so as to spread the suspensions on the medium. The plates were incubated at 30^0 C for 4-5 days.

Isolation of pure culture

The fungi isolated from the soil contain a mixed population exhibiting diverse morphological and physiological characters. Single germinating spores were picked from the mixed culture containing several spores and sub cultured. A pure culture was produced by repeated sub culturing. The purified cultures were then transferred to agar slant and sub cultured fortnightly which was then stored at 4^0 C until use.[9]

Selection of organism based on the utilization of sucrose

The organism present in the pure culture were identified using "Lactophenol cotton blue mounting method", which was especially used for the identification of fungi .The organism used through out the experiment was *Aspergillus and Penicillium* species was selected and identified commonly using 2% glucose medium. This basically depends on their ability to utilize sucrose as their sole carbon source.[9]

Preparation of vegetative inoculum

Aspergillus and *Penicillium* cultures were grown separately on PDA medium for 7 days at room temperature. The slants were then flooded with sterile distilled water and scarped smoothly without disturbing the mycelial growth and filtered through sterile filter. The concentration of the filtrate was adjusted to 10^5 spores / ml and used as inoculum for further studies.[10]

Fermentation technique

Production of β –fructofuranosidase was carried out by shake flask technique using 250 ml Erlenmeyer flasks. Fifty ml of fermentation medium was transferred to each Erlenmeyer flasks. The cotton-plugged flasks were autoclaved at 15 lbs/inch^2 pressure for 15 minutes and cooled in room temperature. A loop of prepared vegetative inoculum from 24 hours old stock culture slants were transferred aseptically to each flask. Flasks were then incubated in a orbitol shaker at 30º C temperature.[11] The sucrose in the medium substituted with sugarcane bagasse which was used as the carbon source.[12]

Processing of the substrate

The sugarcane bagasse was obtained from The Co-operative sugars Ltd, Palakkad. India. It was washed several times in distilled water and then sliced .The sliced pieces were spread on the trays and shade dried The dried slices were ground into fine powder and then sieved .The substrate was stored in the polyethylene bags at room temperature .They were autoclaved at 15 lbs for 20 minutes before use.[13,14]

Analysis and comparison

Dry cell mass of fungal culture was determined by centrifugation of fermentation broth at 5000 rpm using weighed centrifuge tubes. Supernatant was used for further analysis. Residual sugar was estimated by DNS method while reducing sugar releasing activity was assayed with sucrose as substrate by measuring the amount of reducing sugar release by the DNS method. A scanning UV-spectrophotometer was used for the determination of colour intensity at 540nm wavelength. Invertase activity, reducing sugar, and dry cell mass were determined for 6 days at 24 hours interval. Production of invertase by *Pencillium* and *Aspergillus* was assayed in substrates such as sucrose and sugarcane bagasse by the same method.[15]

Optimization of the culture condition

In the industrial exploitation of microbes greater attention is always given to the culture design and standardization of the physiochemical parameter of the medium. Since microorganisms exhibit diverse pattern of nutritional and environmental requirement. The optimization of fermentation medium is done by measuring the enzyme activity by varying the single parameter such as pH, temperature, sucrose (carbon source) concentration, sugarcane bagasse (substituted carbon source) concentration and yeast extract (nitrogen source) concentration of the medium keeping the remaining parameter unaltered.[16,17]

Biomass determination

The mycelial mass was collected by filtration and its wet weight was determined by placing it on a filter paper .The dry weight of each mycelial mass was monitored by drying at 80^0C to constant weight.

Statistical analysis

The Statgraphics statistical package is used for the statistical treatment of data. One-way analysis of variance (Tukey's test, significant level $p < 0.05$) was applied to the data to determine the presence of significant differences in the production of invertase by *Aspergillus fumigatus* and *Penicillium brevicompactum*.

Enzyme assay

β- Fructofuranosidase assay was determined by measuring the reducing sugars released by the hydrolysis of sucrose. The reaction was carried out at 30^0C for 5 minutes. The reducing sugars released in the reaction mixture were assayed by DNS method. The cell free extract obtained after centrifugation is used as the enzyme source for determining the crude enzyme activity.[18]

Enzyme units

One units of invertase (IU) was defined as the amount of enzyme which liberated / μM of product / minute /ml under the assay condition.[19]

RESULTS AND DISCUSSION

The culture conditions directly affect the microbial growth and thereby monitor the metabolic behavior to secrete primary or secondary metabolites. The medium was optimized in order to improve the invertase production by *Aspergillus fumigatus & Penicillium brevicompactum*, using sugarcane bagasse as the carbon source in the medium.

Though invertase can be produced by the use of many substrates, the period of incubation for maximum production is still under question. A comparison of invertase production in shaken flask fermentation by *Aspergillus fumigatus & Penicillium brevicompactum* showed the maximum production at 4th day of incubation. The substrate used for the present study was sugarcane bagasse. Similar result was reported[20] when *Penicillium chrysogenum* was used for the invertase production.

TABLE 1 : Effect of different pH (3-5) with incubation period (48-168Hrs) on invertase production by *Aspergillus fumigatus*

pH	Invertase Activity (IU/ml)					
	Incubation Period (hrs)					
	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr
3	1.73±0.05	2.07±0.40	3.07±0.40	2.71±0.04	2.15±0.06	1.88±0.07
	(1.30)	(1.65)	(2.03)	(2.26)	(2.45)	(2.51)
4	12.45±0.09	16.71±0.04	23.13±0.16	19.43±0.40	10.19±0.17	7.85±0.05
	(1.45)	(1.56)	(1.94)	(2.36)	(2.43)	(2.56)
5	9.60±0.09	15.48±0.08	17.75±0.06	10.64±0.10	10.19±0.17	9.41±0.07
	(1.56)	(1.78)	(1.98)	(2.43)	(2.56)	(2.68)
6	6.40±0.48	12.87±0.08	15.96±0.06	13.23±0.36	8.87±0.10	5.06±0.10
	(1.68)	(1.86)	(1.99)	(2.43)	(2.63)	(2.78)
8	7.18±0.15	11.13±0.04	12.62±0.06	10.10±0.17	8.26±0.10	4.97±0.17
	(1.75)	(1.98)	(2.18)	(2.39)	(2.54)	(2.61)

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The optimum pH of invertase production from *Aspergillus fumigatus* was found to be pH 4.0 at 4th day of incubation, corresponding to 23.08 IU/ml of enzyme activity. (TABLE 1) The pH optimum of invertase from *Penicillium brevicompactum* was found to be 5.0 at 4th day of incubation, corresponding to 18.53 IU/ml of enzyme activity. (Figure 1)

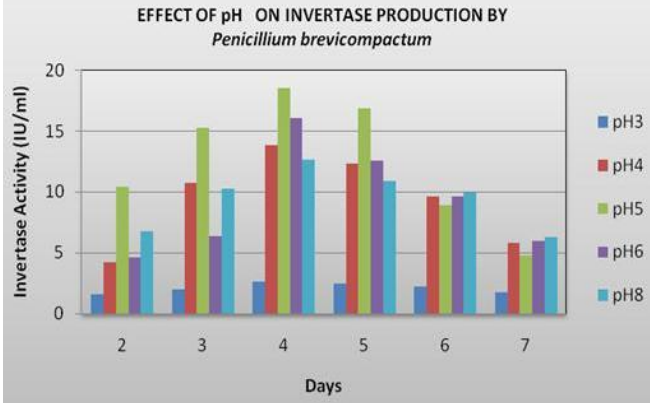

Figure 1 : Effect of different pH (3-5) with incubation period (48-168Hrs) on invertase production by *Penicillium brevicompactum*.

Similar observation was observed using the marine psychrophilic and endemic Antarctic yeast *Leucosporidium antarcticum* 171, for the production of invertase at the optimum pH of 4.0.[21] Studies done by[22] also showed that optimum pH of 5.0 required for the maximum invertase production by *Saccharomyces cerevisiae* IFO 0309.

The optimum temperature of invertase was found to be 30^{0}C for both *Aspergillus fumigatus* & *Penicillium brevicompactum* and corresponding to 20.50 IU/ml and 17.21 IU/ml of enzyme activity. (TABLE 2 and Figure 2)

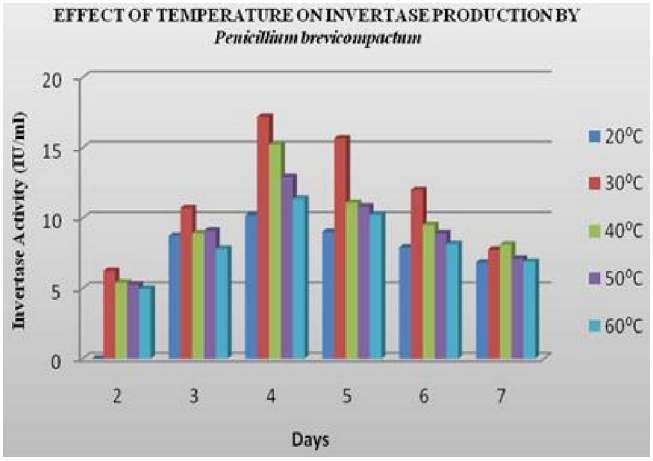

Figure 2 : Effect of different temperature (200C-600C) with incubation period 48-168Hrs) on invertase production by *Penicillium brevicompactum*.

Studies done by[23] showed that optimum temperature for yeast invertase production from *C.utilis* at 30^{0}C by using sauerkraut waste as substrate at shaken flask condition. This observation was also supported by[24], the production of invertase at optimum temperature of 30^{0}C using *Saccharomyces cerevisiae*.

The sucrose concentration on the fermentation medium was varied ranging between 1gm/100ml to 5gm/100 ml. The maximum invertase production from *As-*

TABLE 2 : Effect of different temperature (20^{0}C-60^{0}C) with incubation period (48-168 Hrs) on invertase production by *Aspergillus fumigatus*

Temp. (^{0}C)	Invertase Activity (IU/ml)					
	Incubation Period (hrs)					
	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr
20	5.91±0.12	9.25±0.14	12.63±0.12	10.85±0.11	9.12±0.11	7.63±0.12
	(1.74)	(1.98)	(2.35)	(2.45)	(2.68)	(2.78)
30	8.72±0.11	13.85±0.11	20.50±0.12	17.27±0.11	14.37±0.11	10.74±0.11
	(1.85)	(1.99)	(2.29)	(2.45)	(2.59)	(2.76)
40	8.27±0.13	12.67±0.09	16.79±0.11	14.54±0.11	11.90±0.09	9.65±0.11
	(1.93)	(2.18)	(2.29)	(2.36)	(2.54)	(2.65)
50	7.60±0.11	10.83±0.13	14.23±0.13	12.3±0.18	10.05±0.11	8.33±0.24
	(1.81)	(2.04)	(2.23)	(2.39)	(2.52)	(2.64)
60	6.93±0.11	11.59±0.13	13.46±0.09	11.05±0.13	9.08±0.09	6.27±0.11
	(1.75)	(2.09)	(2.20)	(2.36)	(2.54)	(2.67)

Figures in parenthesis indicates mycelial dry weight (mg)

TABLE 3 : Effect of different concentration of sucrose (1-5gm)/100ml) with incubation period (48-168Hrs) on invertase production by *Aspergillus fumigatus*

Conc. (%)	Invertase Activity (IU/ml)					
	Incubation Period (hrs)					
	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr
1	8.17±0.10	12.74±0.05	18.21±0.07	16.48±0.10	11.16±0.07	6.18±0.16
	(1.45)	(1.56)	(1.69)	(1.96)	(2.11)	(2.18)
2	9.07±0.11	14.88±0.11	23.91±0.51	19.31±0.32	12.69±0.10	7.93±0.26
	(1.36)	(1.49)	(1.68)	(1.83)	(2.09)	(2.19)
3	10.54±0.07	18.23±0.07	25.42±0.23	21.51±0.56	13.11±0.15	9.06±0.21
	(1.48)	(1.61)	(1.86)	(1.99)	(2.25)	(2.36)
4	9.87±0.12	17.19±0.90	24.59±0.94	20.52±0.47	12.87±0.09	8.34±0.23
	(1.37)	(1.44)	(1.59)	(1.93)	(2.20)	(2.42)
5	9.41±0.25	17.28±0.31	24.34±0.45	21.13±0.05	11.21±0.11	7.67±0.02
	(1.39)	(1.59)	(1.76)	(1.94)	(2.16)	(2.35)

Figures in parenthesis indicates mycelial dry weight (mg)

pergillus fumigatus & *Penicillium brevicompactum* was found to be at 3gm/100ml at the 4th day of incubation. (TABLE 3 and Figure 3)

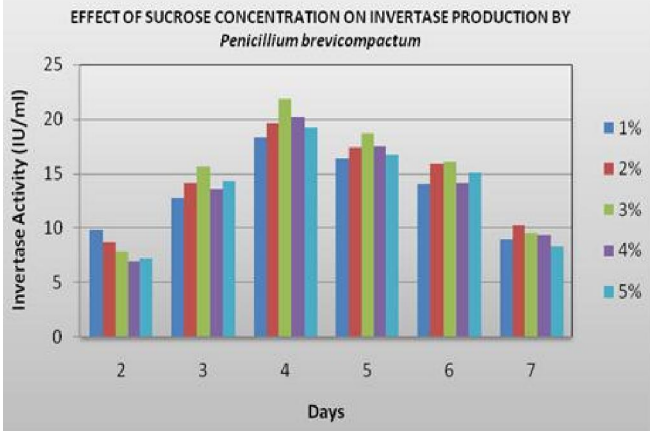

Figure 3 : Effect of different concentration of sucrose (1-5gm/100ml) with incubation period (48-168Hrs) on invertase production by *Penicillium*.

This data was supported by[25] the maximum invertase production was found at sucrose concentration of 30gm/1000ml by *Saccharomyces cerevisiae* GCA-II.

The sugarcane bagasse (substituted carbon source) concentration on the fermentation medium was varied ranging between 1gm/100ml to 5gm/100 ml. The maximum invertase production from *Aspergillus fumigatus* & *Penicillium brevicompactum* was found to be at 4gm/100ml at

TABLE 4 : Effect of different concentration of sugarcane bagasse (Substituted carbon source (1-5gm)/100ml) with incubation period (48-168Hrs) on invertase production by *Aspergillus fumigatus*

Conc. (%)	Invertase Activity (IU/ml)					
	Incubation Period (hrs)					
	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr
1	6.57±0.11	11.35±0.10	18.46±0.11	15.61±0.13	12.36±0.13	10.83±0.07
	(1.13)	(1.26)	(1.36)	(1.45)	(1.68)	(1.95)
2	7.13±0.12	13.73±0.08	20.19±0.08	16.54±0.05	14.81±0.09	17.74±0.06
	(1.20)	(1.26)	(1.36)	(1.45)	(1.59)	(1.86)
3	8.53±0.08	15.63±0.09	22.06±0.10	17.17±0.11	15.02±0.12	11.96±0.11
	(1.36)	(1.48)	(1.56)	(1.76)	(1.89)	(1.93)
4	9.24±0.06	18.43±0.06	24.64±0.09	17.91±0.10	15.27±0.08	12.88±0.09
	(1.36)	(1.53)	(1.76)	(1.83)	(2.01)	(2.16)
5	4.11±0.10	17.93±0.10	23.04±0.12	16.13±0.10	13.72±0.10	11.39±0.09
	(1.52)	(1.73)	(1.98)	(2.09)	(2.19)	(2.31)

Figures in parenthesis indicates mycelial dry weight (mg)

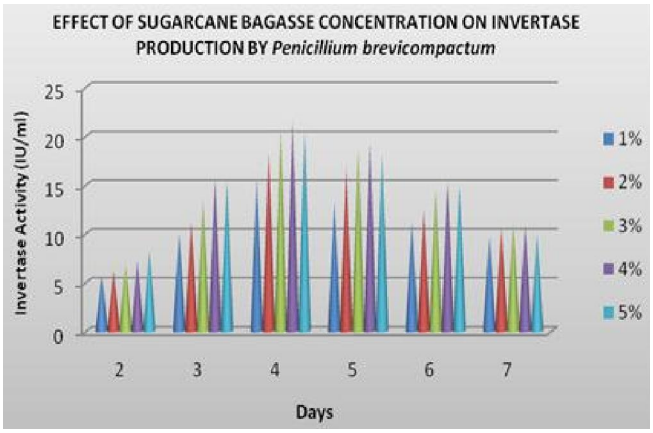

Figure 4 : Effect of different concentration of sugarcane bagasse (Substituted carbon source (1-5gm/100ml) with incubation period (48-168Hrs) on invertase production by *Penicillium brevicompactum*.

the 4th day of incubation. (TABLE 4 and Figure 4) This result obtained was supported by[26] produced high levels of invertase from *A.ochroceus* when cultured for 4th day in khanna medium supplemented with sugarcane bagasse as carbon source.

TABLE 5 : Effect of different concentration of yeast extract (Nitrogen source (0.5-2.5gm)/100ml) with incubation period (48-168Hrs) on invertase production by *Aspergillus fumigatus*

Conc. (%)	Invertase Activity (IU/ml)					
	Incubation Period (hrs)					
	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr
0.5	6.12±0.12	8.71±0.10	12.89±0.11	11.07±0.11	8.26±0.10	6.26±0.10
	(0.78)	(0.99)	(1.35)	(1.45)	(1.59)	(1.78)
1.0	5.19±0.09	9.26±0.10	13.92±0.11	12.20±0.10	9.47±0.11	7.11±0.11
	(0.96)	(1.26)	(1.49)	(1.68)	(1.79)	(1.96)
1.5	6.13±0.12	10.28±0.08	15.11±0.11	13.37±0.11	10.26±0.10	8.50±0.10
	(1.35)	(1.48)	(1.59)	(1.79)	(1.96)	(2.15)
2.0	5.29±0.09	9.97±0.11	14.87±0.11	13.67±0.10	11.58±0.10	9.57±0.10
	(1.52)	(1.68)	(1.79)	(1.89)	(1.97)	(2.04)
2.5	5.74±0.07	10.12±0.12	13.75±0.07	11.68±0.09	9.70±0.10	8.36±0.11
	(1.45)	(1.56)	(1.69)	(1.84)	(2.05)	(2.19)

Figures in parenthesis indicates mycelial dry weight (mg)

The effect of various nitrogen sources on the production of invertase by *Aspergillus fumigatus & Penicillium brevicompactum* species were illustrated. The maximum enzyme activity was found out at 1.5 % yeast extract concentration at the 4th day of incubation for both the organisms. (TABLE 5 and Figure 5) Studies done by[27] also showed the optimum urea concentration as nitrogen source for the

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production of invertase is 3g/1000ml by using *Saccharomyces cerevisiae* GCA-II.

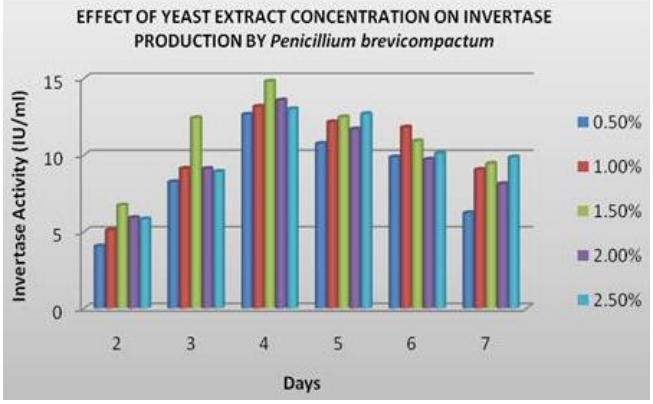

Figure 5 : Effect of different concentration of yeast extract (Nitrogen source (0.5-2.5gm/100ml) with incubation period (48-168Hrs) on invertase production by *Penicillium brevicompactum*.

The result concludes that *Aspergillus fumigatus* produces better results than *Penicillium brevicompactum*. All the parameters analyzed using ANOVA IN TABLE 6 shows the significant difference ($p < 0.05$) in *A.fumigatus* when compared to the *P.brevicompactum.*, so this organism may find industrial application in enzyme fermentation.

TABLE 6 : Comparison invertase production by *Aspergillus fumigatus* and *Penicillium brevicompactum*

PARAMETERS	*Aspergillus fumigatus*	*Penicillium brevicompactum*
pH	23.28 ± 0.24 [a]	18.51 ± 0.25
Temperature	20.43 ± 0.32 [a*]	17.50 ± 0.28
Carbon source (Sucrose concentration)	24.91 ± 0.32 [a*]	21.76 ± 0.21
Substituted carbon concentration	24.64 ± 0.27 [a*]	21.64a ± 0.27
Nitrogen source(Yeast extract concentration)	15.31 ± 0.29 [a*]	14.62 ± 0.19

Data represent the Mean values ± Standard Deviation.
a – *A.fumigatus* is compared with *P.brevicompactum*.
(* - $P < 0.05$)

From the present study, we could see that parameters like pH, temperature, substrate concentration and nitrogen source concentration had an effect in the enzyme production.

In conclusion, *Aspergillus fumigatus & Penicillium brevicompactum* are producers of invertase under the same cultural conditions.

ACKNOWLEDGEMENT

The authors thank the Management of Karpagam University for providing lab facilities and constant encouragement for this research work.

CONTRIBUTION OF AUTHORS

CU analyzed the data and prepared the manuscript. KC collected the samples and performed experimental analysis. VKG coordinated the project and prepared the final manuscript.

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