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Optimization and characterization of thermostable β-glucosidase by *Scytalidium thermophilum* SKESMBKU02

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ABSTRACT

Scytalidium thermophilum SKESMBKU02, a thermophilic fungus isolated from cattle dung, produced a significant amount of thermostable β-glucosidase in Mandel and Weber medium (45°C, 3 days). *Scytalidium thermophilum* SKESMBKU02 opted pH – 5, temperature of 45°C for production of β-glucosidases. The glucose and yeast extract are the best carbon and nitrogen sources for β-glucosidase production. It has found that shake flak culture method exerted more β-glucosidase production than the still culture method. The fungus showed maximum dry weight in cellulose as carbon sources, yeast extract and malt extract as nitrogen source, pH of 9.0-10.0 and temperature of 45°C.The crude enzyme was characterized and found that substrate concentration of 6 mM and enzyme concentration of 300 µl are found suitable for the maximum β-glucosidase activity. Stability studies reveals that the enzyme was active at wide range of pH and temperature.

INTRODUCTION

Cellulose is one of the main components of plant cell wall material and is the most abundant and renewable nonfossil carbon source on earth. Microbial cellulases catalyzing the hydrolysis of β-1,4-glycosidic linkages in plant polysaccharides are industrially important enzymes used to saccharify industrial and agricultural cellulose-containing residues, treat cellulose pulp wastes in the paper industry, enhance the extraction of fermentable substances in the beer brewing and alcohol fermentation industries, etc. (Da-Silva et al., 1997).The degradation of cellulose to glucose is effected by the cooperative action of endocellulases, EG; (EC3.2.1.4), exocellulases (cellobiohydrolase, CBH; (EC3.2.1.74) and β-glucosidases (EC3.2.1.21) (Bhat and Bhat,1997). Endocellulases and exocellulases act synergistically upon cellulose to produce cellobiose, which is then cleaved by β-glucosidase to glucose (Li et al., 2009; Galante et al., 1998).Cellobiose exerts a product inhibition on both CBH and EG (Emert and Brown 1977). BGL not only produces glucose, but also reduces the cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently (Workman and Day, 1982; Ah-Reum Joo et al., 2009). Filamentous fungi are important in industrial enzyme production, since they are able to synthesize and secrete large amounts of extracellular proteins (Oksanen et al., 2000).In recent decades there has been increasing interest in cellulases from thermophilic fungi, which are expected to produce thermostable enzymes. Because of their rapid growth and high rate of cellulose decomposition, thermophilic fungi are an attractive potential source of cellulases. Thermophilic fungi are species that grow at a maximum temperature of 50°C or above, and a minimum of 20°C or above (Maheshwari et al., 2000). The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses (Xue-Cai Hao et al., 2006).The objective of this research was to evaluate the β-glucosidase production by *Scytalidium thermophilum* SKESMBKU02 and to determine the influence of cultural parameters on the activity and stability of this enzyme.

MATERIALS AND METHODS

Maintenance of culture

Thermophilic strains of *Scytalidium thermophilum* SKESMBKU02 used in this study were isolated from cattle dung from Warangal, Telangana, India. It was grown and maintained at 45°C± 2°C on yeast-starch agar medium (YpSs: yeast extract-5gm, starch-15gm, K_2HPO_4-1gm, $MgSO_4$-0.5gm per 1000ml of distilled water). Streptomycin and rose bengal were added to the molten medium after autoclave to inhibit the bacterial growth and the plates were incubated for 3-4 days and maintained on YpSs slants at low temperature (4°C) for further study (Cooney and Emerson, 1964).

Enzyme Production Medium

The 3-4days old culture of *Scytalidium thermophilum* SKESMBKU02 was transferred to production medium described by Mandel and Weber (1969) containing milligrams / liter : $(NH_4)_2SO_4$ - 1,400,KH_2PO_4 - 2,000 ,$CaCl_2 \cdot 2H_2O$ – 300,$MgSO_4 \cdot 7H_2O$ – 300, $FeSO_4 \cdot 7H_2O$ - 5.0, $MnSO_4 \cdot H_2O$ - 1.6, $ZnSO_4 \cdot 7H_2O$ - 1.4, $CoCl_2 \cdot 6H_2O$ - 2.0, Peptone – 100, Tween-80 – 100, pH was adjusted to 5.5 and carbon sources were added at a concentration of 1 percent for all the fermentations. The inoculated medium was incubated at 45°C in shaker incubator for 3, 6,9,12 days. At the end of the fermentation period, the culture medium was filtered through Whatman No.1 filter paper to obtain the crude extract, which served as enzyme source. Fermentation was carried out in duplicate and average values from duplicates are presented in this work.

Assay of β-D-Glucosidase activity

Activity of β-D-glucosidase in the culture filtrate was quantified according to the method of Herr (1979). Its activity was measured in assay mixture containing 0.2 ml of 5 mM para-nitrophenyl β-D-gluco pyranoside (pNPG) solution and 0.7 ml of citrate phosphate buffer (0.05 M, pH 5.5) and 0.1 ml of diluted enzyme solution with appropriate controls. After incubation for 30 min at 60°C, the reaction was stopped by adding 4 ml of 0.25 M NaOH-glycine buffer (pH 10.6). The yellow colored p-nitrophenol liberated was determined at 420 nm. One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1 µmole of p-nitrophenol per min under standard assay conditions.

Effect of initial and final medium pH on β-glucosidase production.

The effect of pH on β-glucosidase production was assessed by cultivating *Scytalidium thermophilum* SKESMBKU02 in 150ml flask containing 25ml of optimized media of varied pH range from 3.0-10.0, the pH of the medium was adjusted by using 1N HCl or 1N NaOH and incubated at 45°C in shaker incubator for 3, 6,9,12 days at regular intervals the enzyme was extracted and used as β-glucosidase source (Roberto et al., 2005).

Effects of temperature on β-glucosidase production.

The optimum temperature for the β-glucosidase production by *Scytalidium thermophilum* SKESMBKU02 was carried out by incubating the production media containing carbon sources 1% (glucose) and nitrogen source 0.2% ($(NH_4)_2SO_4$), pH of 5.5 and different temperature (35°C - 55°C) for 3,6,9,12days (Gomes et al.,2000).

Effect of carbon source on β-glucosidase production

The effect of different carbon sources on production of β-glucosidase was studied (glucose, fructose, maltose, lactose, xylose, starch, carboxy methyl cellulose, cellulose and sucrose (10 g/l).The optimized media were inoculated with *Scytalidium thermophilum* SKESMBKU02 and incubated in an orbital shaker at the optimum agitation rate (150 rpm). At the end of incubation time the enzyme activity in cell-free culture supernatant was assayed. (Coronel et al., 1991)

Effect of nitrogen source on β-glucosidase production

The effect of nitrogen sources on β-glucosidase production was assayed by various nitrogen source including peptone, yeast extract, malt extract, beef extract, urea, $(NH_4)_2 SO_4$, $NaNO_3$, KNO_3, NH4Cl tested at a concentration of (0.2% w/v) inoculated with 3-4day old culture of *Scytalidium thermophilum* SKESMBKU02 incubated in an orbital shaker at agitation rate of 100 rpm for 3,6,9,12 days. At the end of incubation time the enzyme activity was assayed (El-Hadi et al., 2014).

Effect of still and shake flask condition on β-glucosidase production

The effect of still and shake flask condition on β-glucosidase production was studied by incubating the production media inoculated with *Scytalidium thermophilum* SKESMBKU02 and kept still and shake flask conditions (100,150,200rpm) for 3,6,9,12 days. At the end of incubation time the enzyme activity was carried out as mentioned above (Shahriarinour et al., 2013).

Determination of fungal biomass

At regular intervals of time (3, 6,9,12 days) the contents of the flasks were aseptically passed through pre-weighed Whatman No.1 filter paper to separate mycelial mat from culture filtrates. The filter papers, along with mycelial mat were dried at 70°C in an oven for overnight and weight was recorded. The difference between the weight of the filter paper bearing mycelia mat and weight of pre-weighed filter paper represented fungal biomass, which was expressed in terms of dry weight of mycelia mat in milligrams (Srivastava et al., 2011).

Characterization of crude enzyme

Scytalidium thermophilum SKESMBKU02 were grown in production media for 3days contents of the flasks were aseptically passed through Whatman No.1 filter paper to separate mycelial mat from culture filtrates. The supernatant was used as crude enzyme source for enzyme characterization by varying the concentration of enzyme from 50-500 µl, after incubation enzyme activity was determined by enzyme assay. Substrate concentration was determined by varying the concentration of substrate from 2-10µM (para-nitrophenyl β-D-gluco-pyranoside (pNPG)) remaining activity was determined by enzyme assay (Sujatha et al., 1995). The pH stability (pH 3.0 to10.0) of the crude enzyme was evaluated by mixing the enzyme and buffer to give final proportion of 0.1:0.7 (v/v). These solutions were incubated at 45°C for 1 hour and remaining activity was determined by enzyme assay (Ravindran et al., 2010). Thermal stability measurement for the enzyme was done by incubating the crude enzyme with in the temperature range of 35°C - 55°C for 1hr, and then quickly cooling the mixture in an ice-water bath (4°C). The remaining activity was measured by enzyme assay at 60°C for 30 min (Quiroz et al., 2009).

RESULTS AND DISCUSSION

Effects of pH on β-glucosidase production

Transportation of various chemicals across the cell membrane, including movement of enzyme and their activity is importantly influenced by the pH of the medium (Santosh et al.,2014). Current finding also shows that pH of production medium was an important factor affecting β-glucosidase production. Table 1 illustrates that the initial β-glucosidase activity increased with the increase of pH and the peak activity was at found at pH 6, while further increase in pH showed decreasing trend in β-glucosidase activity. It has been reported that the optimal pH for fungal cellulases varies from species to species. Joo et al., (2009) reported that Fomitopsis pinicola KMJ812 opted pH- 5 for cellulases production in submerged fermentation which are in agreement with the findings of *Scytalidium thermophilum* SKESMBKU02.

Table 1: Effects of pH on β –glucosidase production by *Scytalidium thermophilum* SKESMBKU02

Effect of pH	Days of Incubation	*Scytalidium thermophilum* SKESMBKU02		
		pH	Dry. Wt. (mgs)	β –glucosidase activity/U/ml
pH 3	3	3.04	60	ND
	6	2.65	80	ND
	9	2.20	100	0.010
	12	2.00	120	0.005
pH 4	3	3.99	60	ND
	6	3.32	70	ND
	9	3.00	100	0.015
	12	2.56	120	0.006
pH 5	3	5.26	40	**0.068**
	6	4.67	50	0.015
	9	4.35	60	0.010
	12	4.00	80	0.006
pH 6	3	5.43	40	0.064
	6	4.38	60	0.038
	9	4.15	100	0.016
	12	3.82	120	0.010
pH 7	3	5.03	30	0.036
	6	4.26	50	0.017
	9	3.00	90	0.01
	12	2.88	110	ND
pH 8	3	4.80	30	0.008
	6	4.10	40	0.006
	9	3.86	60	ND
	12	3.48	90	ND
pH 9	3	5.04	80	0.006
	6	4.20	110	0.003
	9	3.65	130	ND
	12	3.19	210	ND
pH 10	3	5.16	50	ND
	6	4.46	100	ND
	9	4.00	140	ND
	12	3.69	180	ND

ND = No activity Detected

Effects of temperature on β-glucosidase production

The effect of temperature on β-glucosidase production was studied by growing the organism at different temperature. As the fermentation temperature was increased from 35°C to 55°C cell growth and β-glucosidase activity in the culture supernatant gradually increased (Table 2). After 50°C, the β-glucosidase activity gradually decreased. Since the maximum β-glucosidase activity was observed at 45°C, this temperature was selected in the subsequent experiments to determine the effect of other parameters for growth and β-glucosidase production. The findings are in agreement with the Hayashida, and Yoshioka (1980), who reported that Humicola insolens YH-8 opted temperature of 50°C for the production of cellulases.

Table 2: Effects of temperature on β –glucosidase production by *Scytalidium thermophilum* SKESMBKU02

Effect of temperature (in °C)	Days of Incubation	*Scytalidium thermophilum* SKESMBKU02		
		pH	Dry. Wt. (mgs)	β –glucosidase activity/U/ml
35 °C	3	5.35	30	0.023
	6	5.15	60	0.015
	9	3.50	80	0.003
	12	2.65	100	ND
45 °C	3	4.82	50	0.068
	6	4.25	60	0.023
	9	3.35	90	0.013
	12	3.00	110	0.006
50 °C	3	5.40	50	0.020
	6	4.95	90	0.010
	9	3.30	170	0.003
	12	3.00	200	ND
55 °C	3	5.30	50	0.015
	6	5.25	60	0.010
	9	3.80	150	ND
	12	2.85	190	ND

Table 3: Effects of carbon sources on β –glucosidase production by *Scytalidium thermophilum* SKESMBKU02

Carbon sources	Days of Incubation	*Scytalidium thermophilum* SKESMBKU02		
		pH	Dry. Wt. (mgs)	β –glucosidase activity/U/ml
Glucose	3	4.82	30	**0.068**
	6	4.20	70	0.031
	9	3.83	80	0.012
	12	3.15	100	0.003
Xylose	3	5.21	40	0.007
	6	4.82	60	0.015
	9	4.00	80	0.010
	12	3.83	90	ND
Cellulose	3	5.30	20	0.022
	6	5.00	60	0.029
	9	4.84	130	0.016
	12	4.00	150	ND
Starch	3	5.03	30	0.035
	6	3.81	60	0.019
	9	3.00	80	0.010
	12	2.91	100	ND
CMC	3	4.50	20	0.022
	6	4.20	30	0.029
	9	4.00	40	0.013
	12	3.30	80	ND
Sucrose	3	5.13	70	0.015
	6	4.93	80	0.010
	9	4.85	90	0.003
	12	4.55	100	ND
Maltose	3	5.00	70	0.015
	6	4.86	90	ND
	9	4.73	90	ND
	12	4.36	100	ND
Fructose	3	4.56	70	0.006
	6	4.30	80	ND
	9	4.05	100	ND
	12	4.00	130	ND
Lactose	3	5.08	70	0.010
	6	4.87	90	0.006
	9	4.36	100	ND
	12	4.00	120	ND

ND = No activity Detected

Effect of carbon source on β-glucosidase production

To study the effect of different carbon sources on β -glucosidase production, a number of carbon sources at a concentration of 1.0% were tested (Table 3). Glucose was the best amongst the carbon sources tested. Starch and other carbon sources produced lower levels of β -glucosidase activity. The effect of carbon sources on β -glucosidase production by these fungi supports the findings of Shahzadi et al., (2014) who found that β-glucosidase activity was more in glucose containing production media.

Table 4: Effects of nitrogen sources on β –glucosidase production by *Scytalidium thermophilum* SKESMBKU02

Nitrogen sources	Days of Incubation	*Scytalidium thermophilum* SKESMBKU02		
		pH	Dry. Wt. (mgs)	β –glucosidase activity/U/ml
Peptone	3	4.85	80	0.025
	6	4.55	90	0.030
	9	4.20	90	0.020
	12	3.00	100	0.009
Yeast extract	3	5.20	30	0.060
	6	5.00	50	**0.150**
	9	4.86	70	0.055
	12	4.20	80	0.020
Malt extract	3	4.13	100	0.020
	6	4.00	110	0.030
	9	4.00	130	0.025
	12	3.79	140	0.015
Beef extract	3	5.00	60	0.025
	6	4.15	80	0.055
	9	4.00	90	0.035
	12	3.56	110	0.012
Urea	3	4.83	40	0.015
	6	4.50	60	0.020
	9	4.30	80	0.015
	12	4.00	100	ND
$(NH_4)2SO_4$	3	4.93	100	0.012
	6	4.50	110	0.015
	9	4.30	120	0.002
	12	4.00	130	ND
$NaNO_3$	3	5.00	60	0.024
	6	4.83	80	0.020
	9	4.55	100	0.016
	12	4.00	110	0.003
KNO_3	3	5.15	60	0.012
	6	4.53	80	0.019
	9	4.20	100	0.010
	12	3.40	110	0.005
NH_4Cl	3	5.20	70	0.019
	6	5.00	90	0.016
	9	4.65	110	ND
	12	4.15	130	ND

ND = No activity Detected

Table 5: Effects of still and shake flask on β –glucosidase production by *Scytalidium thermophilum* SKESMBKU02

Effect of shake flask (RPM)	Days of Incubation	*Scytalidium thermophilum* SKESMBKU02		
		pH	Dry. Wt. (mgs)	β –glucosidase activity/U/ml
100	3	4.82	50	0.068
	6	4.25	60	0.029
	9	3.35	80	0.003
	12	3.00	90	0.010
150	3	4.80	50	0.015
	6	4.42	90	0.010
	9	3.70	100	0.006
	12	3.00	120	ND
200	3	4.30	50	0.012
	6	3.90	60	0.010
	9	3.40	90	ND
	12	2.88	110	ND
Still condition	3	5.25	50	0.018
	6	5.00	60	0.016
	9	3.50	90	0.007
	12	2.65	110	0.003

ND = No activity Detected

Effect of nitrogen source on β-glucosidase production

To investigate the effect of nitrogen sources on β-glucosidase production *Scytalidium thermophilum* SKESMBKU02 was grown in production media and found that the highest production of β-glucosidase was in media containing yeast extract and malt extract. The yeast extract proved best and gave maximal production of β-glucosidase (Table 4). The nitrogen sources are the powerful factors regulating the synthesis of enzymes by fungi. The results are in agreement with the findings of Shahriarinour etal.,(2011) who found that β-glucosidase activity is more in organic nitrogen source than the inorganic nitrogen sources.

Effect of still and shake flask condition on β-glucosidase production

The production of β-glucosidase was studied in still and shakes flask condition (Table 5). In general, cellulase production was increased in shake flask condition, this might be explained by the fact that the shaking speed increased the dissolved oxygen in the culture, which is necessary for cell membrane components and uniform distribution of the medium contents such as nutrients (Rajagopalan and Krishnan, 2008). In the static culture, a layer of mycelium grew at the top of the culture, not evenly distributed which significantly reduced the contact time and area between the fungal cells and substrates. In cultures agitated at 100 rpm, growth of *Scytalidium thermophilum* SKESMBKU02 was about 3 times higher than those obtained in static culture.

Determination of fungal biomass

The biomass production of *Scytalidium thermophilum* SKESMBKU02 signifies that biomass increased with incubation period. The results disclose that the *Scytalidium thermophilum* SKESMBKU02 showed increased biomass in pH of 9.0 – 10 (Table 1) and temperature of 50°C (Table 2). Cellulose found to be best carbon sources for biomass production followed by fructose and lactose (Table 3).Among nitrogen sources malt extract and NH4Clwere found to produce maximum fungal biomass (Table 4). Compare to the still, maximum fungal biomass was produced in the shake flask culture at 150 RPM (Table 5).There is no co-relationation between β-glucosidase production and biomass production.

Characterization of crude enzyme

A systematic characterization of β-glucosidase from thermophilic fungi is necessary to better understand the activity of enzyme at different concentrations of enzyme, substrate and stability studies. β-glucosidase activity was more at enzyme concentration of 300 µl (Figure.1), optimum substrate concentration found to be 6.0mM (Figure. 2), optimum pH,temperature for β-glucosidase activity was found to be pH – 5.0 and 65° C respectively (Figure. 3,4). The results are in harmony with the findings of Sujatha et al., (2006) who found that the activity of crude enzyme is influenced by the substrate and enzyme concentration

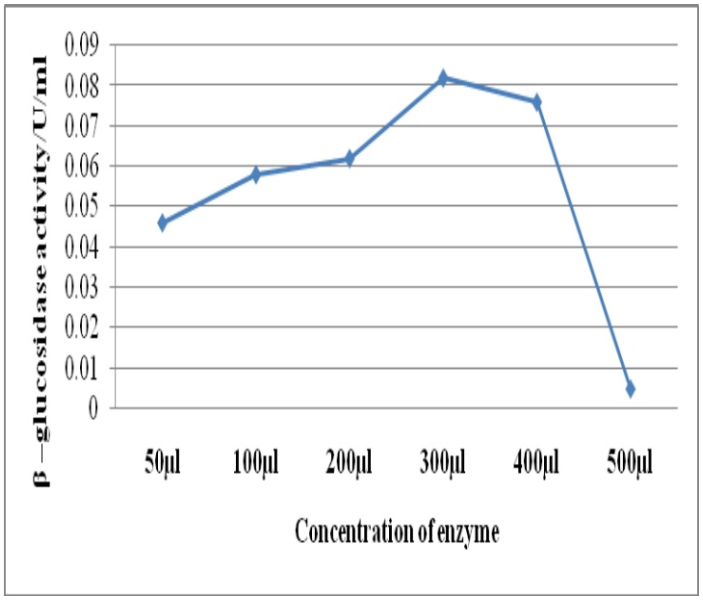

Fig.1: Effect of enzyme concentration on β-glucosidase activity

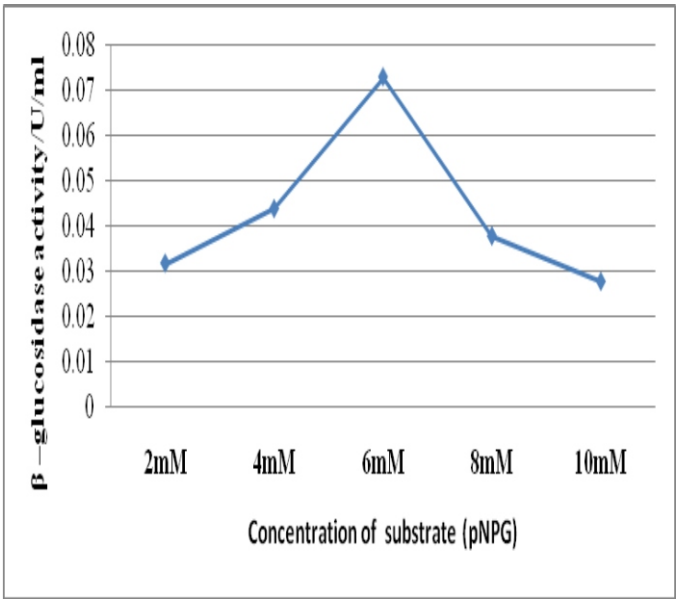

Fig.2: Effect of substrate concentration on β-glucosidase activity

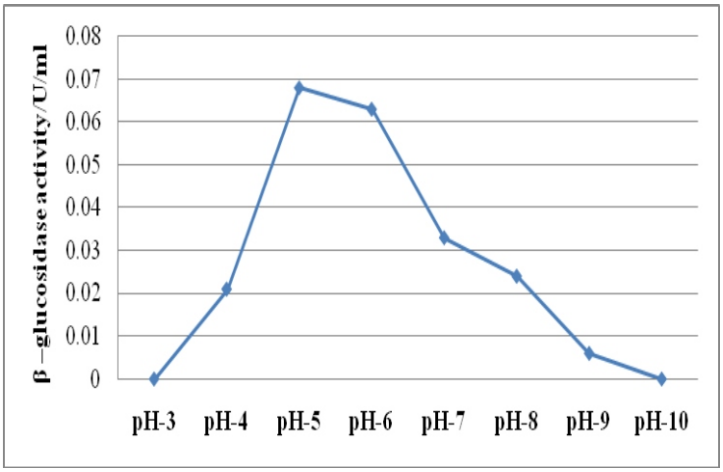

Fig.3: Effect of pH on stability of β-glucosidase activity

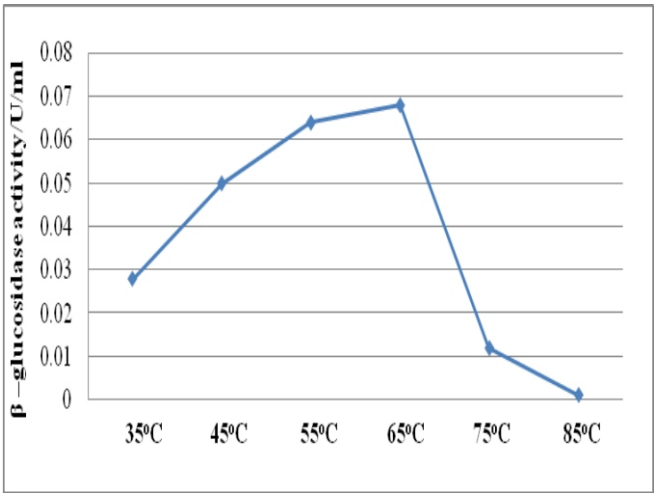

Fig.4: Effect of temperature on stability of β-glucosidase activity

CONCLUSION

The result has enabled the ideal cultural composition for maximum β-glucosidase production by *Scytalidium thermophilum* SKESMBKU02. The high activity and stability of β-glucosidase between neutral and alkaline pH and high temperature will be of use in various industrial and biotechnological applications.

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