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REGULAR ARTICLE

PRODUCTION OF AN EXTRACELLULAR CELLOBIASE IN SOLID STATE FERMENTATION

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ABSTRACT

The bioethanol production from lignocellulosic biomass has attracted wide interest globally in last decade. One of the main reasons for the high cost of bioethanol production from lignocellulosic biomass is the expensive enzymes involved in enzymatic hydrolysis of cellulose (cellulase). The utilization of agro-industrial waste as a potential substrate for producing enzymes may serve a dual purpose of reducing the environmental pollution along with producing a high value commercial product. Twelve different agro-industrial wastes were evaluated for extracellular cellobiose or β-glucosidase production by a mutant of *Bacillus subtilis* on solid state fermentations (SSF). The *Citrus sinensis* peel waste was found to be the most suitable substrate with highest BGL titre (35 U/gds). Optimum incubation time, inoculum size, moisture content and volume of buffer for enzyme extraction were 72 h, 40 % v/w, 10 mL and 20 mL respectively.

Keywords: *Bacillus subtilis*, solid state fermentation, cellobiase, *Citrus sinensis*, mutant, biofuels

INTRODUCTION

There is a growing interest globally in producing ethanol from different lignocellulosic biomasses (agriculture residue, perennial crops, woody substance and municipal solid waste). These are mainly composed of cellulose, hemicellulose and lignin, and may serve as cheap and renewable feedstocks for bioethanol production. Cellulose is made up of uniform structure of β-1,4 linked glucose units and its biodegradability may vary in different biomasses depending on the strength of association of the cellulose with other plant compounds. The cost of enzymatic hydrolysis of biomass varies from 15 - 30% of the total cost of biofuel production. An important enzyme cellobiase or β-glucosidase (BGL) catalyzes the hydrolysis of alkyl, aryl-β-glucosides, diglucosides and oligosaccharides. This is a key enzyme for cellulose degradation and is widely used in the production of fuel ethanol, prevent discoloration of fruit juices, cause enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermenting products **(Shoseyov *et al.*, 1990; Das *et al.*, 2004; Zhang *et al.*, 2006)**. BGL is a part of the cellulase complex. It is inhibited by glucose, has high susceptibility to thermal inactivation and also lower BGL concentration leads to catabolite repression due to accumulation of cellobiose **(Ikram-ul-Haq *et al.*, 2006)**. It is therefore desirable to have a higher extracellular BGL production. So, the focus is on various agro-industrial waste that can be utilized to produce BGL enzyme by microbial transformations in an eco-friendly and economic way. With the purpose of reducing the cost of the culture media we selected different wastes or substrates for the maximum extracellular production of BGL.

Several agro-industrial wastes such as sweet sorghum peel waste, rice straw, rice bran, wheat straw, wheat bran, *Citrus sinensis* peel waste, tea leaves extract, filter paper, banana peel, popular wood, sugarcane bagasse and corn cobs are produced all over the world. *Citrus sinensis* is a citrus fruit produced all over the world including India with maximum production being from Maharashtra, Tamil Nadu, Andhra Pradesh, Himachal Pradesh, Punjab and Haryana. Citrus fruit waste (peel, pulp and seed) disposed by fruit-processing industries (that produce squashes, pickles, marmalades etc.) attract flies and rats leading to serious pollution issues. Also, the ruminants do not feed on this particular waste. Benefits like low cost of availability, high carbon and nutrient content and less storage constraints make a substrate highly suitable for BGL production by solid-state fermentations (SSF) **(Ali *et al.*, 2010; Bhardwaj *et al.*, 2010; Dhillon *et al.*, 2011)**. SSF in turn is less expensive and the benefits like direct applicability of the product, the high product concentration, and the reduced costs

of dewatering make SSF an attractive technology for BGL production **(Vandevoorde and Verstraete, 1987; Krishna 1999)**.

The objective of our study was to obtain and optimize an economic culture media for high and commercially viable extracellular BGL yields on SSF. In this paper, several agro-industrial by-products were tested as substrates for BGL production by a hyperproducer strain produced by mutating *Bacillus subtilis*.

MATERIAL AND METHODS

Micro-organism

A BGL hyperproducing mutant strain of *B. subtilis* (PS-5CM-UM3), originally isolated from sugarcane bagasse and mutated by combined ultraviolet and chemical mutagenesis, was used in this study **(Agrawal *et al.*, 2012)**. The Luria Bertani agar media (HiMedia, Mumbai, India) was used for the culture growth.

Inoculum preparation

A 10 mL Luria broth was sterilized at 121°C for 20 min, cooled and inoculated under aseptic conditions with loopful culture of *Bacillus subtilis* mutant, PS-5CM-UM3. The broth culture was incubated for 20 h on a rotary shaker (120 rpm) at 37°C till Optical Density at λ_{600nm} reached upto 0.7-0.8. The cell suspension was used for inoculating the solid media.

Substrate preparation

Twelve agro-wastes (sweet sorghum peel waste, rice straw, rice bran, wheat straw, wheat bran, *Citrus sinensis* peel waste, tea leaves extract, filter paper, banana peel, popular wood, sugarcane bagasse and corn cobs) were collected from Pantnagar, India. These were oven dried at 50°C for 48 h and broken into small pieces. These pieces were then ground and stored in air-tight glass bottles at room temperature (30°C) until use.

BGL production in SSF

Erlenmeyer flasks (250 mL) containing 5 g of agro-wastes and moistened with 10 mL of the mineral salt medium (g/L: $Na_2HPO_4.2H_2O$, 1.3; $NaH_2PO_4.2H_2O$, 0.8; KCl, 0.32; $MgSO_4.7H_2O$, 0.03; Tween-80, 1%; pH 7.0) were autoclaved at 121°C for 20 min. 2 mL inoculum was added after cooling to about 30°C. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and incubated in slanting position at 37°C for 72 h. The incubator was humidified by a tray with sterile distilled water (relative humidity of 60±65%). The enzyme was extracted by adding 30 mL (1:6 w/v ratio) aceate buffer (0.1 M, pH 5.0) to each flask, with a contact time of 30 min and agitation at 150 rpm at 37°C on a rotary shaker. The contents were centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant obtained was finally filtered through Whatman filter paper no. 1 and assayed for BGL activity and reducing sugar content.

BGL assay

BGL activity was assayed by incubating the reaction mixture containing 10 mM p-nitrophenyl-β-D-glucopyranoside (pNPG) and culture filtrate in 1: 1 ratio for 1 hour at 60°C. The reaction was stopped by adding 2 mL of 1 M Na_2CO_3. The p-nitrophenol release was monitored at λ_{405nm} in UV-Visible spectrophotometer. One unit (U) of BGL was defined as the amount of the enzyme to produce one µmole p-nitrophenol per minute and reported on the basis of gram dry substrate (U/gds) used in the SSF, under the assay conditions.

Optimization of process parameters for higher BGL production on *Citrus sinensis* peel waste

Various process parameters that influence the BGL production during SSF (viz. incubation period, inoculum size, minimal salt solution content and buffer volume to extract the enzyme) were optimized over a wide range. The effect of an individual parameter was evaluated at each step and incorporated at the optimized level in the next consecutive step.

Effect of incubation period on SSF with *Citrus sinensis* peel waste

Eight separate cultures were prepared with 5 g of *Citrus sinensis* peel waste having 10 mL of minimal salt solution and 2 mL inoculum. All sets were kept at 37°C for different

incubation periods (12, 24, 36, 48, 60, 72, 84 and 96 h). Enzyme was extracted with 30 mL of actate buffer and BGL titre was checked.

Effect of inoculum size on SSF with *Citrus sinensis* peel waste

A variation in inoculum sizes (1, 2, 3, 4 and 5 mL) was made and BGL titre was monitored. Culture conditions were adjusted to 5 g of *Citrus sinensis* peel waste, 10 mL of minimal salt solution, 37°C incubation temperature, 72 h of incubation time and 30 mL buffer was used for BGL extraction.

Effect of volume of minimal salt solution on SSF with *Citrus sinensis* peel waste

BGL production was studied with different volumes of minimal salt solution (2, 4, 6, 8, 10 and 12 mL). Culture conditions were 5 g of *Citrus sinensis* peel waste, 2 mL inoculum, 37°C incubation temperature, 72 h time of fermentation (incubation time) and 30 mL buffer for enzyme extraction and BGL titre was monitored.

Effect of buffer volume used for extraction of enzyme during SSF on Citrus sinensis peel waste

Using 5 g of *Citrus sinensis* peel waste, 10 mL of minimal salt solution, 2 mL inoculum, 37°C incubation temperature, 72 h time of fermentation for each set, optimal volume of buffer required for enzyme extraction was assessed. Enzyme was extracted with different volumes (10, 20, 30, 40 and 50 mL) of acetate buffer (0.1 M, pH 5.0) and BGL titre was checked.

Statistical analysis

For statistical analysis, standard deviation for each experimental results was calculated using Excel spread-sheets available in Microsoft Excel. Results represented in this study are means of three independent determinations.

RESULTS AND DISCUSSION

BGL activity on different agro-industrial wastes

Bacillus subtilis mutant (PS-5CM-UM3) produced maximum BGL enzyme (41.44 U/gds) by SSF when *Citrus sinensis* peel waste was used as the solid substrate (Table 1). Wheat straw gave only 7.45 U/gds which is about $1/5^{th}$ of the enzyme yield produced on *Citrus sinensis* peel waste. The BGL production on sweet sorghum peel waste, rice straw, rice bran, wheat bran, tea leaves extract, filter paper, banana peel, popular wood, sugarcane bagasse and corn cobs powder was negligible as compared to that on the *Citrus sinensis* peel waste. This difference in the substrate utilization by the bacteria may be because of the difference in the substrates composition. This preference in utilizing one (*Citrus sinensis* peel waste) than other (banana peel or corn cobs) may be attributed to better anchorage of the bacteria to that substrate (Dhillon *et al.*, 2004).

Table 1 Comparative cellobiase titres produced by utilizing different agro-industrial wastes (substrates) in SSF system

Substrate	U/gds ± SD
Sweet sorghum peel waste	0.39 ± 0.78
Rice bran	2.75 ± 0.78
Rice straw	0.39 ± 0.78
Wheat bran	0.39 ± 0.78
Wheat straw	7.45 ± 0.78
Citrus sinensis peel waste	41.44 ± 1.63
Tea leaves extract	0.39 ± 0.78
Filter paper	0.39 ± 0.78
Banana peel powder	2.75 ± 1.56
Popular wood powder	0.39 ± 1.56
Sugarcane bagasse	2.48 ± 1.63
Corn cobs powder	0.65 ± 0.45

Culture conditions: Minimal salt solution: 10 mL, pH: 7.0; Incubation temperature: 37°C; Inoculum size: 2 mL; Time of fermentation (incubation time): 72 h, Buffer volume for enzyme extraction: 30 mL, SD = standard deviation

Effect of incubation time on SSF

Citrus sinensis peel waste was chosen as an ideal substrate and different parameters were optimized. Optimum incubation time was 72 h with 41.18 U/gds and a drop in enzyme titer was observed after further incubation (figure 1). Prolonged incubation would have led to depletion of nutrients, cell death, proteolytic digestion or this may be because of the denaturation of the enzymes at varied pH which is a common phenomenon during fermentation due to the release of acidic by-products in the media **(Dhillon *et al.*, 2004)**.

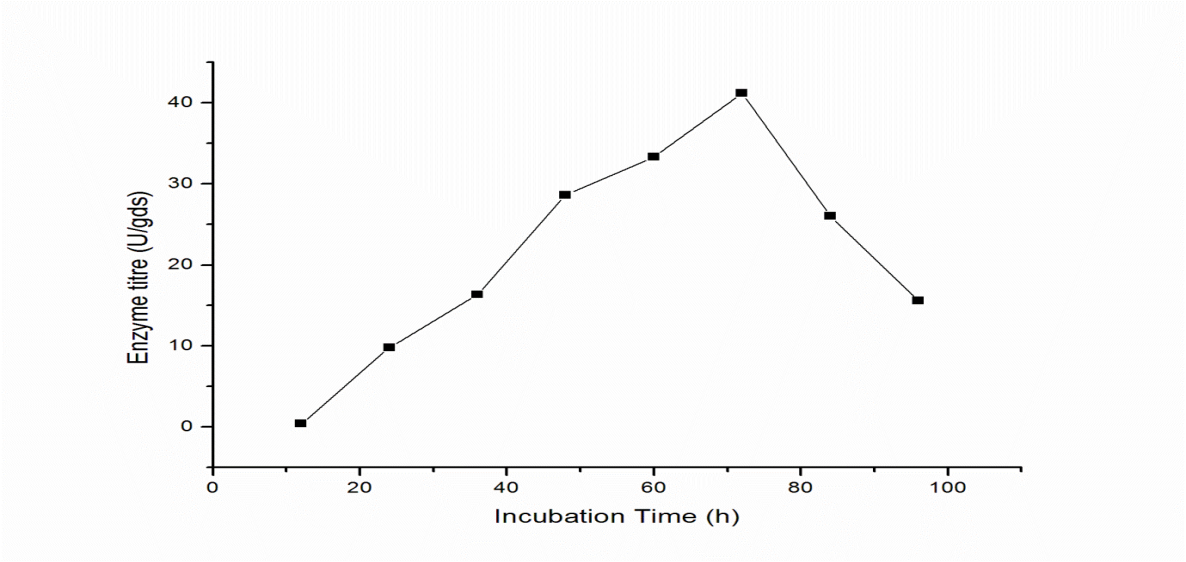

Figure 1 Effect of incubation time on BGL titres in a SSF system (*Citrus sinensis* peel waste with mineral salt medium without any supplement) Culture conditions: Minimal salt solution: 10 mL, pH: 7.0; Incubation temperature: 37°C; Inoculum size: 2 mL, Buffer volume for enzyme extraction: 20 mL

Effect of inoculum size and minimal salt solution content on SSF

Optimum inoculum size and minimal salt solution content were found to be 2 mL and 10 mL respectively (Fig. 2 and Fig. 3). Higher inoculum sizes decreased the BGL production which may be attributed to decreased surface area to volume ratio and an increase in the competition among the biomass for the nutrients. Distribution of dissolved oxygen and proper nutrient uptake is also hindered by higher inoculum sizes. Low size of the inoculum may instead lead to insufficient amount of the BGL producing bacteria. Minimal salt solution content is highly detrimental in BGL production as it strongly affects the concentration gradient between the hydrolyzing enzymes and available carbohydrates present in the

substrate. It eventually causes swelling of the substrate, better substrate utilization and eased exchange between particles and the bacteria **(Kim *et al.*, 1985; Vandevoorde, and Verstraete, 1987; Dhillon *et al.*, 2004)**.

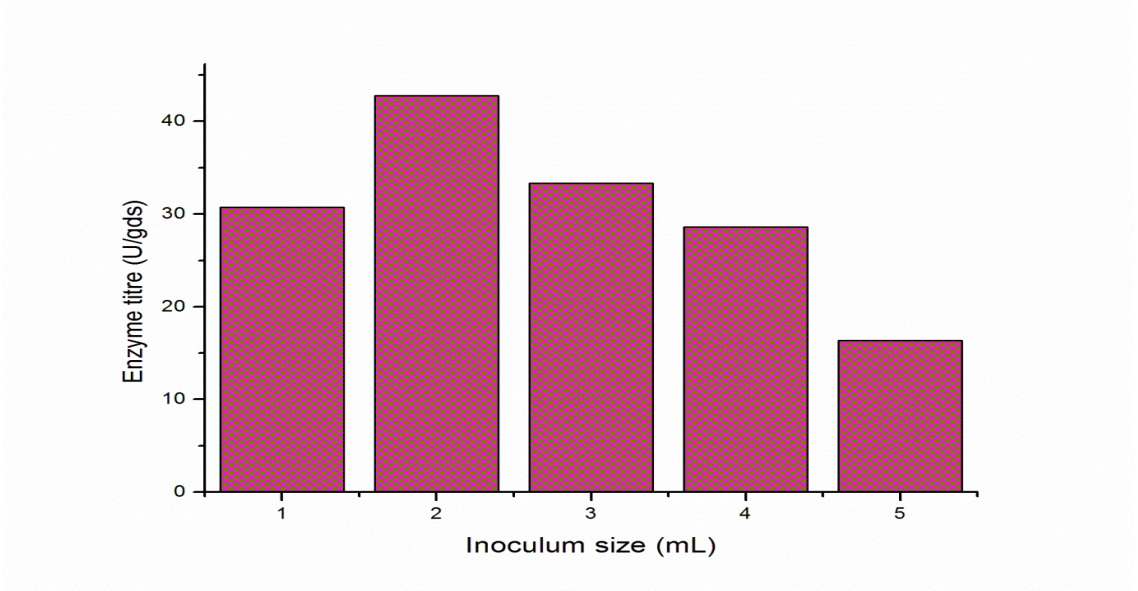

Figure 2 Effect of inoculum size on BGL titres in a SSF system (*Citrus sinensis* peel waste with mineral salt medium without any supplement) Culture conditions: Minimal salt solution: 10 mL, pH: 7.0; Incubation temperature: 37°C; Time of fermentation (incubation time): 72 h, Buffer volume for enzyme extraction: 20 mL

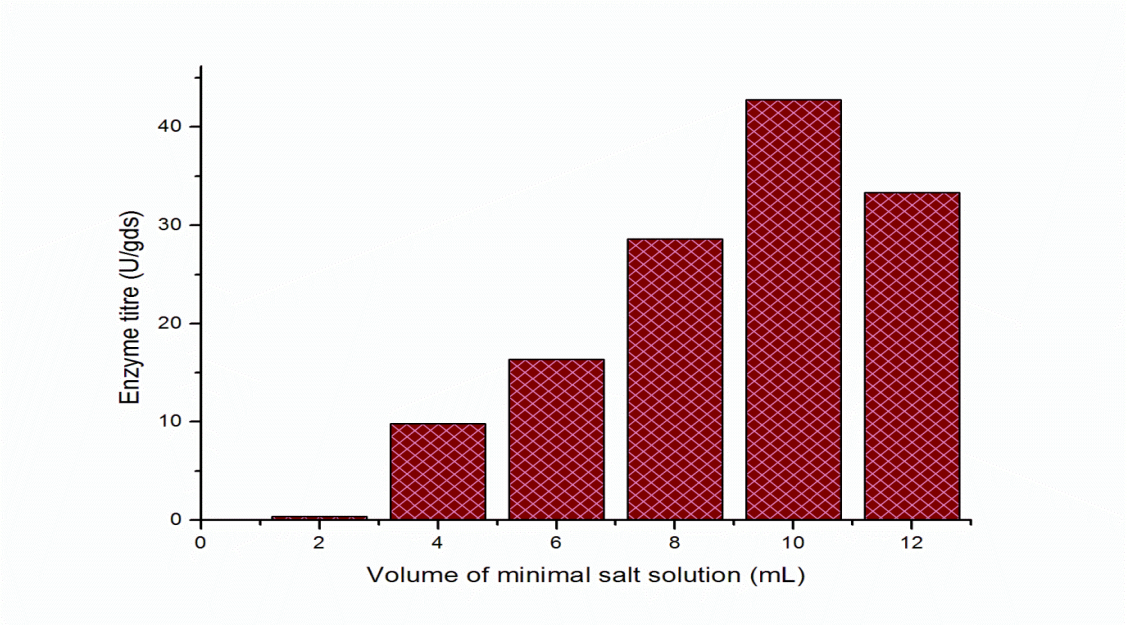

Figure 3 Effect of minimal salt solution content on BGL titres in a SSF system (*Citrus sinensis* peel waste with mineral salt medium without any supplement) Culture conditions: pH: 7.0; Incubation temperature: 37°C; Inoculum size: 2 mL; Time of fermentation (incubation time): 72 h, Buffer volume for enzyme extraction: 20 mL

Effect of buffer volume on SSF

An increasing trend in the enzyme yield was observed when various parameters were further optimized. After optimization of the volume of buffer added to extract the enzyme (20 mL) BGL titre reached up to 84.44 U/gds (Figure 4). Excessive use of buffer for extraction process must have diluted the enzyme yielding low enzyme titer while scanty use of the buffer affected the proper extraction of the enzyme.

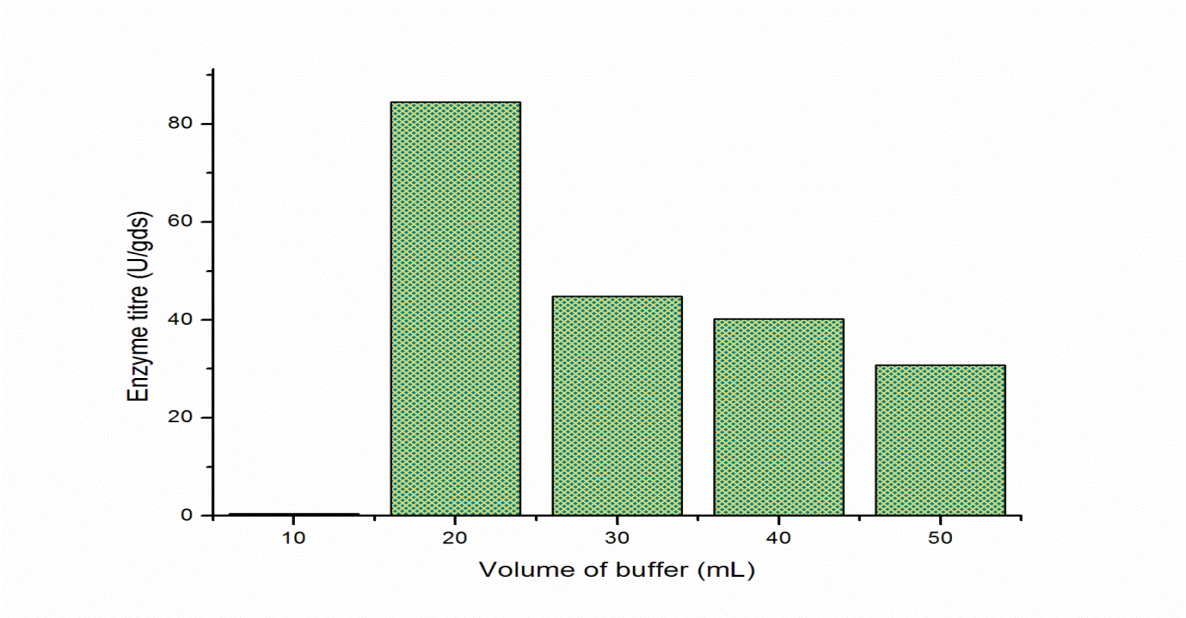

Figure 4 Effect of buffer volume on BGL titres in a SSF system (*Citrus sinensis* peel waste with mineral salt medium without any supplement) Culture conditions: Minimal salt solution: 10 mL, pH: 7.0; Incubation temperature: 37°C; Inoculum size: 2 mL; Time of fermentation (incubation time): 72 h

Optimization of incubation temperature

The activity of of the partially purified β-glucosidase enzyme at different temperatures was monitored. The enzyme displayed maximal activity at 60 °C (Fig. 5). This is in contrast to the β-glucosidases isolated from corn stover which showed maximal activity around 37 °C, and showed depressed activity below 30 °C and above 40 °C. A total loss in activity was observed at 60 °C for 100 min and 70 °C for 1 min Temperature stability of β-glucosidase from other sources has been reported to be ranging from 50 °C to 65 °C **(Christakopoulos *et al*. 1994; Yan and Lin, 1997; Yazdi *et al*. 2003; Karnchanatat *et al*. 2007)**.

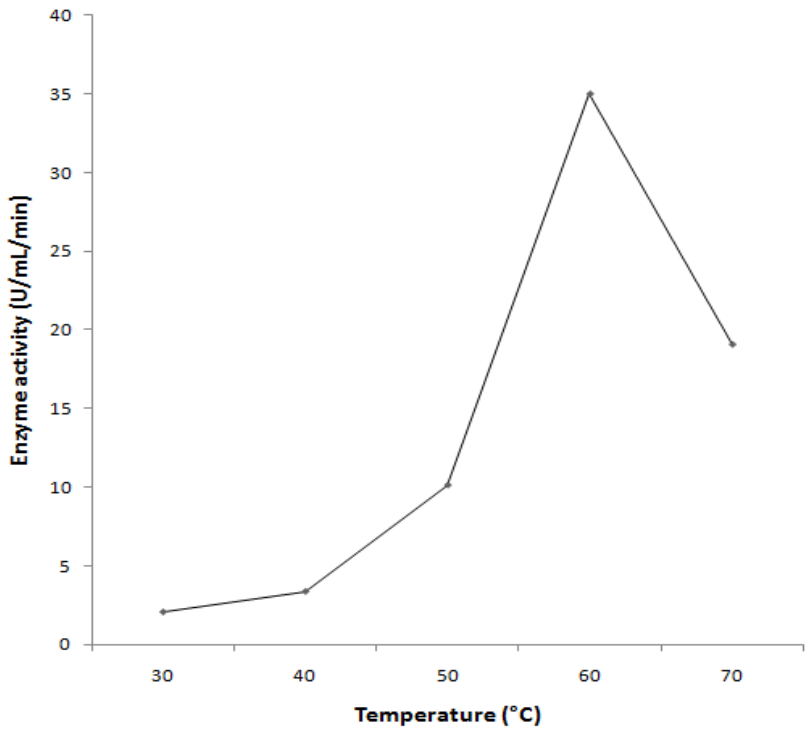

Figure 5 Optimization of incubation temperature for purified β-glucosidase

CONCLUSION

This study demonstrates that cheap agro-industrial wastes can be utilized for the production of costly commercial enzymes. This shows an ecofriendly way to remediate the biomass waste. The production of a cheaper thermostable cellobiase could be useful for the biofuel industry.

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