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Research Article

Isolation and Screening of Cellulase Producing Thermophilic Bacteria from Compost Piles and Optimization of Cellulase Production

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ABSTRACT

The cellulase producing bacteria were isolated from compost pile collected from Navagam, District- Navsari, Gujarat, India. Total 5 isolates were obtained by the primary screening technique from which 2 isolates were showing maximum cellulase activity. These 2 isolates were then evaluated by secondary screening for enzyme production. These 2 isolates T1 and T2 were selected as most efficient enzyme producers and their specific enzyme activity in the crude sample was found to be 0.52 U/mg and 0.59 U/mg and of partially purified sample was found to be 0.20 U/mg and 0.18 U/mg respectively. Isolates were tentatively characterized on the basis of their morphological, cultural and biochemical characteristics, T1 and T2 both were identified to be *Bacillus* species. Further partial purification of the cellulase enzyme was carried out by ammonium sulphate precipitation followed by dialysis. Optimization of different parameters was carried out for the production of cellulase by both efficient isolates T1 and T2.

Keywords: Thermophilic cellulolytic bacteria, Compost pile, Cellulase, Partial purification, Optimization, Bacillus sp.

[I] INTRODUCTION

Microbial species exist in many environments like extremes of temperature, pH, chemical content and pressure. This existence of microbes is due to certain genetic or physiological adaptation [9]. The Extremophiles include; Halophiles, Thermophiles, Basophiles, Psychrophiles and Acidophiles [3]. Thermophiles are the organisms which are adapted to live at high temperatures [5]. The search for extremophilic organisms is one of the means for obtaining enzymes with properties suitable for industrial applications [17]. Since most industrial processes are carried out at high temperatures, therefore there is a great demand for thermophilic enzymes [14]. In search for thermophilic bacteria compost pile samples were taken because it is hot inside, usually the temperature of the central portion of the compost pile reaches up to 80° C due to heat emitted as a result of microbial respiration [32].

Cellulose is the most abundant biomass on the earth. Cellulose is commonly degraded by an

enzyme called cellulase [24]. Microorganisms; fungi, actinomycetes and bacteria produce cellulase during their growth on cellulolysic material [4, 18]. Bacteria has high growth rate as compared to fungi has good potential to be used in cellulase production [16]. Production of various biologically active metabolites *i.e.* antibiotics, enzymes and bacteriocins by the thermophilic bacteria make them attractive candidates for biological control [31]. These thermo-tolerant microorganisms have shown tremendous potential in biotechnology because of their thermoactivity and thermo stability as compared to that of mesophilic organisms [8, 9, 21]. Bacterial genera such as *Cellulomonas sp, Pseudomonas sp., Bacillus sp, Micrococcus sp.* were reported for having celluloytic property [16, 24]. Certain cellulase producing bacteria are also inhabiting the Erthworm gut which are responsible for decomposition of organic matter and composting [29].

Cellulases are inducible enzymes which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials [24] which can degrade and hydrolyze lignocellulosic and cellulosic waste [11, 30]. Cellulases have attracted much interest because of diversity of their application such as textile, food, detergent, leather and paper industries [2], fibre modification, Waste Management, Wine and Brewery, Animal Feed, Agriculture and pharmaceutical industries [7, 16]. Besides, cellulases are also used in ruminant nutrition for improving digestibility, in fruit juices processing and another emerging application is de-inking of paper [27]. These industrial applications focused on to the cellulases which can be highly stable and in active state at extreme pH and temperature.Cellulose is biologically renewable resource abundantly found in agriculture waste. The cellulosic waste material can be hydrolysed to glucose and other soluble sugars by using cellulase enzymes of bacteria. The reducing sugars obtained can be further used for the production of ethanol as biofuel [10].

There are quite a few advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes [17]. The main advantage is that as the temperature of the process is increased, the rate of reaction increases. A 10 ~ increase in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed [14]. The thermostable enzymes are also able to tolerate higher temperatures, which give a longer half-life to the enzyme. The use of higher temperatures (above 60 ~ also is inhibitory to microbial growth, decreasing the possibility of microbial contamination [2]. Cellulase yields appear to depend on a variety of factors like inoculum size, pH, temperature, incubation period, presence of inducers, medium additives, aeration, and growth time. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be improved [16, 28].

The present study was attempted with the following objectives:

- To isolate and screen thermophilic cellulolytic bacteria from different compost piles.
- Production of cellulase by potential isolates by submerged fermentation process.
- Partial purification of cellulase and determination of its Enzyme activity and Specific activity.
- Optimization of different parameters for better cultivation and production process.

[II] MATERIALS AND METHODS

2.1. Sample collection, Isolation and Primary screening for Cellulase producing thermophilic bacteria

From 5 different sites, total 15 Samples were taken from the middle region of domestic compost pile (located in Navagam, District-Navsari, Gujarat, India) where the temperature

was ≥50°C which was measured by using compost thermometer. The samples were collected in sterile container and stored at 4ºC until used. For enrichment, Compost pile samples were inoculated in complete medium broth (Peptone 2.5g, K_2HPO_4 0.1g, KH_2PO_4 0.15g, Yeast extract 0.75g, Beef extract 0.75g, NaCl 2.5g, Glucose 2.5g, $MgSO_4.7H_2O$ 0.05g, distilled water 500ml, pH -7) [26] and incubated at high temperature 60°C, 70° C, 75°C and 80°C till visible turbidity was observed. Tenfold serial dilutions of each compost pile sample were prepared in sterilized distilled water and 0.1 ml of each dilution was spreaded on modified complete agar medium (Peptone 2.5g, K_2HPO_4 0.1gm, KH_2PO_4 0.15g, Yeast extract 0.75g, Beef extract 0.75g, NaCl 2.5g, Glucose 2.5g, $MgSO_4.7H_2O$ 0.05g, Agar – 6g, distilled water 500ml, pH -7). For aerobic thermophilic bacteria plates were kept in closed container to preserve the humidity and double concentration of agar in media preparation was used to avoid dehydration of agar. The plates were incubated at 60°C, 70°C, 75°C and 80°C till sufficient isolated colonies were observed. After the appearance of the colonies, they were observed for morphological and colonial characteristics**.** These isolated thermophilic bacteria were then screened for their cellulase activity. The primary screening of thermophilic isolates for cellulase activity was carried out using Carboxymethylcellulose (CMC) agar medium ($(NH_4)_2SO_4$ 0.7g, KH_2PO_4 1.0g, $MgSO_4.7H_2O$ 0.15g, $CaCl_2$ 0.15g, $FeSO_4.7H_2O$ 0.0025g, $MnSO_4.H_2O$ 0.0068g, Protease Peptone 0.5g, Urea 0.15g, $ZnCl_2$ 0.00085g, CMC 5g, Distilled water 500ml, Agar 30g, pH 7). The plates were incubated at 75˚C for 48–78 hrs. Following incubation, the plates were flooded with 1% Congo red and 1M NaCl to see the cellulolytic activity of isolated strain. The formation of a clear zone of hydrolysis indicated the cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase producer. The largest ratio was assumed to contain the highest activity. All the potent isolates were then preserved on sterile Nutrient agar slants under refrigeration for further study [13].

2.2. Secondary screening and production of cellulase enzyme

The potential thermophilic isolates T1 and T2 were then evaluated for enzyme productivity. Those isolates showing maximum cellulase production were then considered for the further study [28].

2.2.1. Submerge Fermentation process

For preparation of standard inoculum, those isolates showed a maximum zone of hydrolysis were cultured in 20 ml inoculum medium [Composition (g/l): Carboxymethylcellulose (CMC) 5, Tryptone 2, KH_2PO_4 4, Na_2HPO_4 4, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.001, $FeSO_4.7H_2O$ 0.004 and pH adjusted to 7] individually and incubated at 37 ºC for 24 hours. This was used as inoculum for the production medium. The composition of production medium was same as of inoculum medium except the concentration of Carboxymethylcellulose which was 1% instead of 0.5%. Fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile production medium and inoculated with 5% of standard inoculums (containing 2-3.5x10^6 cells/ml). The flasks were incubated at 37 ºC on a rotary shaker at 150 RPM for 72h.

2.2.2. Preparation of crude enzyme

After incubation, the cultures were centrifuged at 1600 RPM for 20 min at 4°C and supernatant was used as a source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activity.

2.3. Cellulase enzyme assay

Carboxymethylcellulase activity was estimated using a 1 % solution of Carboxymethylcellulose in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 1ml of

crude enzyme solution. The reaction was carried out at 45°C for 30 min. The amount of reducing sugar released in the hydrolysis was measured by DNSA method [22]. The Enzyme unit (EU) was determined as the amount of CMCase required to release 1µmole of reducing sugar per ml per minute under above assay condition [25].

2.4. Protein determination

Protein concentrations in a crude sample were determined by using a Folin Lowry's method [20] with bovine serum albumin (BSA) as a standard.

2.5. Partial purification of cellulase enzyme

2.5.1. Ammonium sulfate precipitation

About 20 ml of the crude enzyme solution was saturated by solid ammonium sulphate and the mixture was left overnight at 40°C for precipitation [19].

The precipitates were collected by centrifugation and dissolved in 10 ml of 50 mM sodium acetate buffer (pH 5.5).

2.5.2. Dialysis

For partial purification, enzyme collected after ammonium sulfate precipitation was dialyzed against 30mM sodium acetate buffer (pH- 5.5) at 4ºC with three changes of buffer [19]. The partially purified sample was assayed for enzyme activity and protein content.

2.6. Identification of cellulase producing bacteria

Potential isolates were tentatively identified by means of morphological, cultural and biochemical characterization as per Bergey's Manual [28].

2.6.1. Morphological characterization

For morphological characterization colonies were stained by Gram's staining technique and for suspected isolates special staining was also performed such as endospore staining.

2.6.2. Cultural characterization

The pure cultures of most efficient isolates were passed on modified complete agar plates. The plates were incubated at 75°C for 48 hrs. After incubation colony characteristics were noted.

2.6.3. Biochemical characterization

Different biochemical tests were analyzed included Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, starch hydrolysis, gelatin liquefaction, nitrate reduction, Catalase test, Oxidase test, Phenylalanine deamination and sugar fermentation test.

2.7. Optimization of various parameters for maximum cellulase activity

The optimum parameters were determined for cellulase production from the efficient isolates. The cellulase fermentation was carried out at different ranges of parameters included temperature, pH, incubation period, nitrogen source, substrate concentration.

After fermentation at different parameters the crude enzyme sample was collected from each to check the enzyme activity [25].

2.7.1. Effect of temperature

To determine the optimum temperature for cellulase production, fermentation was carried out at various temperatures in the range of 60°C, 70°C, and 75°C.

2.7.2. Effect of pH

Different values of pH ranged from 6 to 8 were chosen for studying their effects on cellulase enzyme production.

2.7.3. Effect of incubation period

To obtain maximum cellulase production, fermentation was carried out at different incubation periods ranging from 48, 72, 96 and 120 hours**.**

2.7.4. Optimization of Nitrogen source

To obtain maximum cellulase production, fermentation was carried out by using different nitrogen sources such as Ammonium Phosphate, Sodium Nitrate and Urea at the concentration of 1%.

2.7.5. Effect of Substrate

Three different substrates such as CMC, wheat straw and wood strips were used to evaluate the optimum production of cellulase.

2.7.5.1. Carboxymethylcellulose (CMC)

To evaluate the effect of concentration of CMC on cellulase production, the production medium was supplemented with different concentration of CMC including 0.5%, 1.0%, 1.5%, 2.0%.

2.7.5.2. Wood strips and Wheat straw

Wood strips and Wheat straw were subjected to chemical pre-treatment and evaluated the effect of this pre-treatment on the Cellulase enzyme activity.

This study helped to identify effective pre-treatment and the relative importance of individual pre-treatment on the rate of enzymatic hydrolysis of the cellulosic material [25].

2.7.5.2.1. Effect of Pre-treatment with Acid

To determine the optimum concentration of acid for pre-treatment of substrates for maximum cellulase activity, Substrates were added into conical flask containing 100ml of different concentrations of acid (1%, 0.3%, 0.5%, 0.7%, 0.9%, 1%, 1.1%, and 1.3%). The flasks were left at room temperature for 24 hours.

The substrates were then neutralized with NaOH and auto autoclaved at 121ºC for 15 minutes at 15 lbs. Once the flasks had cooled, they were inoculated with T1 and T2 at the rate of 1% Inoculum and then incubated on a rotary shaker with 120 rpm at 75°C.

2.7.5.2.2. Effect of Pre-treatment with Alkali

To determine the optimum concentration of alkali for pre-treatment of substrates for maximum cellulase activity, Substrates were added into conical flask containing 100ml of different concentrations of alkali (1%, 0.3%, 0.5%, 0.7%, 0.9%, 1%, 1.1%, and 1.3%). The flasks were left at room temperature for 24 hours. The substrates were then neutralized with Hcl and auto autoclaved at 121ºC for 15 minutes at 15 lbs. Once the flasks had cooled, they were inoculated with T1 and T2 at the rate of 1% inoculum and then incubated on a rotary shaker with 120 rpm at 75°C.

[III] RESULT AND DISCUSSION

3.1. Isolation and primary screening for cellulase producing thermophilic bacteria

Total 5 thermophilic bacterial isolates T1, T2, T3, T4 and T5 were obtained. From these, T4 and T5 were removed due to their morphological and colonial characteristics. The resulting 3 isolates T1, T2 and T3 were then tested on CMC agar for cellulase activity. Their CMCase activity is shown in Table 1. Among them T1 and T2 gave the maximum ratio of clear zone diameter to colony diameter on the CMC agar plate as compared to plate cultured with T3. The efficient thermophilic cellulase producers T1 and T2 then analyzed for secondary screening.

Sr. no.	Isolate no.	Colony diameter (n) (mm)	Zone diameter (z) (mm)	Zone of clearance (z/n) (mm)
1	T1	4.3	8.2	**2.05**
2	T2	4.0	7.2	**1.67**
3	T3	3.8	5.2	1.37

mm– millimetre

Table 1. Zone of hydrolysis of thermophilic isolates

3.2. Secondary screening and production of cellulase enzyme

On the basis of primary screening, the potential isolates were then evaluated for their enzyme productivity in submerge fermentation process. For the enzyme activity study, both crude enzyme and partially purified enzyme samples were assayed by cellulase enzyme assay method.

3.3. Enzyme activity assay

The protein concentration in crude samples was determined with bovine serum albumin (BSA) as standard. The Enzyme unit (EU) of both crude and partially purified enzyme was determined by

using DNSA method and their specific activity was calculated which is listed in Table 2.

Isolate no.	Specific activity (U/mg of protein)	
	Crude enzyme	Partially purified enzyme
T1	0.52	0.20
T2	0.50	0.18

U- Unit, mg- milligram

Table 2. Specific activity of enzyme from thermophilic isolates

3.4. Identification of most efficient cellulase producing thermophilic bacteria

T1 and T2 both were tentatively identified to be endospore forming *Bacillus sp.* on the basis of their morphological, cultural and biochemical characteristics [6, 15]. Their morphological, colonial and biochemical characteristics are tabulated in Table 3 and 4.

Isolate no.	Colony characteristics		Growth characteristics on special media (Potato slice medium)	Morphology
	On nutrient agar plate	On Mac Conkey's agar plate		
T1	Large, Irregular, Entire, raised, dry, opaque, non-pigmented colony	-	Creamy white dry growth observed	Gram positive thick rods arrange in chain of two cells, presence of central endospore.
T2	Large, Irregular, Entire, raised, dry, opaque, non-pigmented colony	-	Creamy white dry growth observed	Gram positive, slender short rods, occurring singly, presence of subterminal endospore

Table 3. Colony and morphological characteristics of most efficient isolates

Biochemical tests	T1	T2
Indole production	+	+
Catalase	+	+
Oxidase	+	+
Methyl red	+	+
Voges-Proskauer	+	+
Citrate utilization	+	+
Nitrate reduction	+	+
Phenylalanine deamination	-	-
Gelatin liquefaction	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Ammonia production	+	+
Sugar fermentation		
Glucose	⊕	⊕
Lactose	⊕	⊕
Maltose	+	+
Xylose	⊕	⊕
Mannitol	⊕	⊕
Sucrose	-	-
Fructose	⊕	⊕
Ribulose	-	-
Arabinose	-	-

(-) Negative, (+) Positive and ⊕ Presence of acid and gas

Table 4. Biochemical characteristics of isolates

3.5. Optimization of cellulase production

The optimum parameters were determined for cellulase production from the efficient isolates. After fermentation at the different parameters the crude enzyme product was collected for determination of enzyme activity. Enzyme activity was determined by DNSA method. The enzyme activity of T1 and T2 at the different parameters is tabulated in Table 5.

Data illustrated in fig. (1) Clearly indicated that the highest enzyme activity of T1 and T2 was found to be 0.41U/ml and 0.32 U/ml respectively at 70 °C. However the optimum temperature for cellulase activity produced by other thermophilic *Bacillus subtilis* isolated from another compost pile from Nepal was 50°C [1]. Like temperature, pH and incubation period is also an important factor that influences the cellulase yield. The results illustrated by fig. (2) Clearly show that cellulase production, expressed as enzyme activity, gradually increased as the pH values increased from 6 to 7 and reached to maximum at pH of 8 for both T1 and T2 being 0.45 & 0.47 U/ml of cellulase respectively. Studies by A. Acharya *et al.* [1] suggest pH 7.2 for maximum enzyme activity for *Bacillus sp*. and according to Duffaud *et al.* [12], bacterial cellulases are active at alkaline pH. The incubation period of 96 hours achieved the highest cellulase enzyme production which is illustrated in fig. (3). As illustrated in fig. (4) Urea was found to better nitrogen source as compared to other nitrogen sources and the enzyme activity was 0.23U/ml&0. 20U/ml for T1 and T2 respectively. Carboxymethylcellulose was found to better substrate as compared to others. The 1.0 % concentration of Carboxymethylcellulose results in an increase in enzyme activity of T1 and T2, being 0.61 U/ml and 0.57 U/ml respectively fig. (5). Results illustrated by Fig. (6, 7, 8, 9) Clearly show that the cellulase enzyme activity for both T1 and T2 was found to be maximum when wheat straw and wood strip were pretreated with at 1.0% of acid and alkali. Studies by Prasad M.P. and Sethi R. [25] suggest pre-treatment is an essential prerequisite to enhance the Cellulase production and activity. According to tentative analysis, both T1 and T2 were found to be *Bacillus sp.* These data indicated that T1 was more efficient cellulase producer as compared to T2. The data obtained in this study can be used to model the process parameters to make economical evaluations of the cellulase production process.

Different parameters	Different values	Enzyme activity (U/ml)	
		T1	T2
Temperature	60°C	0.38	0.30
	70°C	**0.41**	**0.32**
	75°C	0.35	0.27
pH	6	0.21	0.25
	7	0.31	0.31
	8	**0.45**	**0.47**
	9	0.43	0.36
Incubation period	48hr	0.31	0.29
	72hr	0.42	0.34
	96hr	**0.51**	**0.47**
	120hr	0.50	0.45
Nitrogen source (1%)	Ammonium Nitrate	0.17	0.19

	Ammonium Sulphate	0.11		0.13	
	Ammonium Phosphate	0.12		0.14	
	Urea	**0.23**		**0.20**	
Substrate					
Carboxymethylcellulose	0.5%	0.49		0.43	
	1.0%	**0.61**		**0.57**	
	1.5%	0.52		0.51	
	2.0%	0.45		0.33	
Wheat straw	Concentration of acid and alkali (%)	Pre-treatment with acid	Pre-treatment with alkali	Pre-treatment with acid	Pre-treatment with alkali
	0.1	0.12	0.06	0.09	0.09
	0.3	0.12	0.08	0.09	0.09
	0.5	0.14	0.10	0.10	0.12
	0.7	0.16	0.13	0.12	0.12
	0.9	0.17	0.17	0.16	0.14
	1.0	**0.22**	**0.21**	**0.19**	**0.19**
	1.1	0.21	0.21	0.18	0.18
	1.3	0.21	0.18	0.15	0.14
Wood strip	0.1	0.11	0.09	0.10	0.08
	0.3	0.12	0.09	0.10	0.09
	0.5	0.12	0.11	0.12	0.10
	0.7	0.14	0.13	0.13	0.13
	0.9	0.16	0.17	0.16	0.17
	1.0	**0.21**	**0.24**	**0.18**	**0.21**
	1.1	0.21	0.24	0.17	0.20
	1.3	0.19	0.20	0.16	0.16

U- Unit, ml- millilitre

Table 5. Optimization of cellulase production

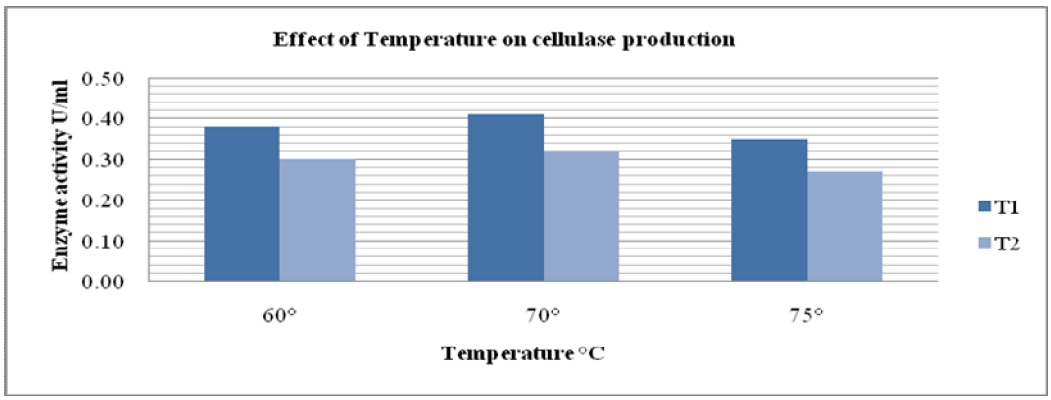

Figure 1. Effect of temperature on cellulase production by T1 and T2

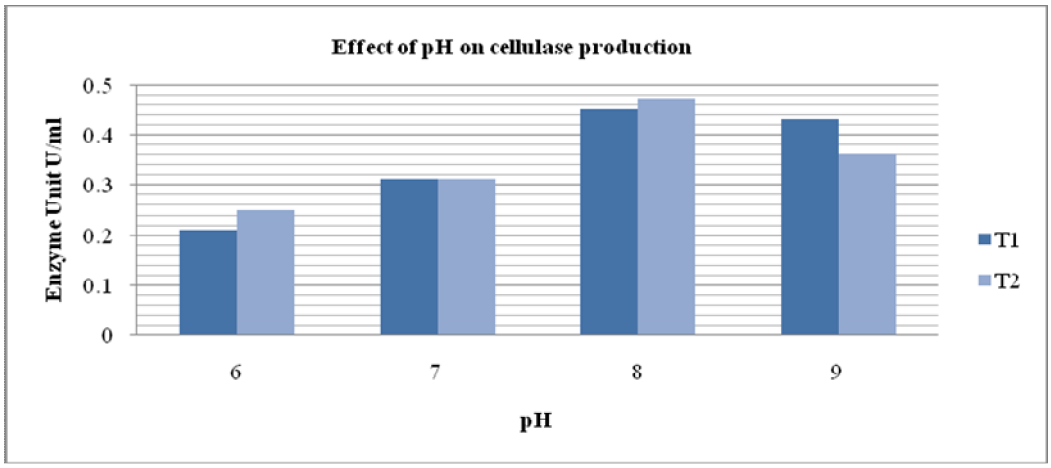

Figure 2. Effect of pH on cellulase production by T1 and T2

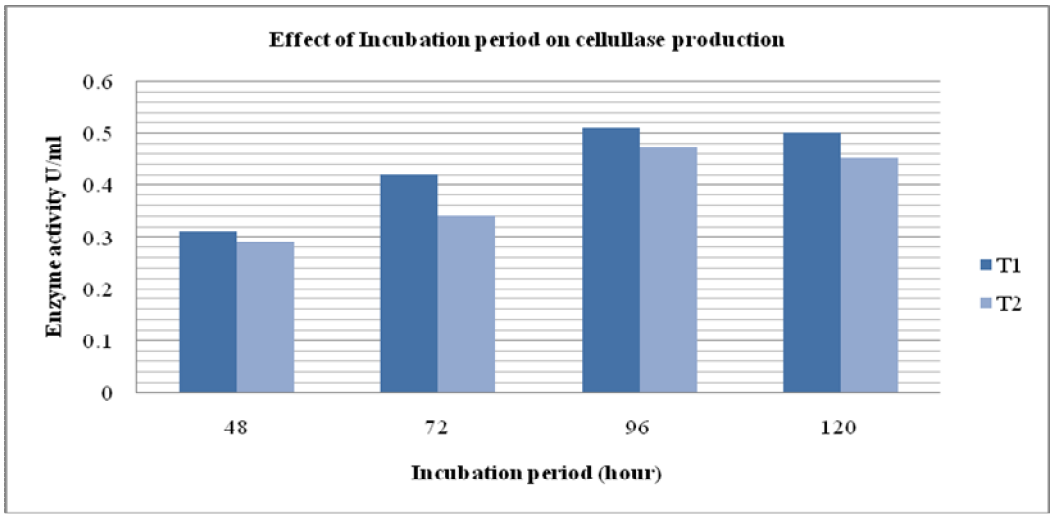

Figure 3. Effect of Incubation period on cellulase production by T1 and T2

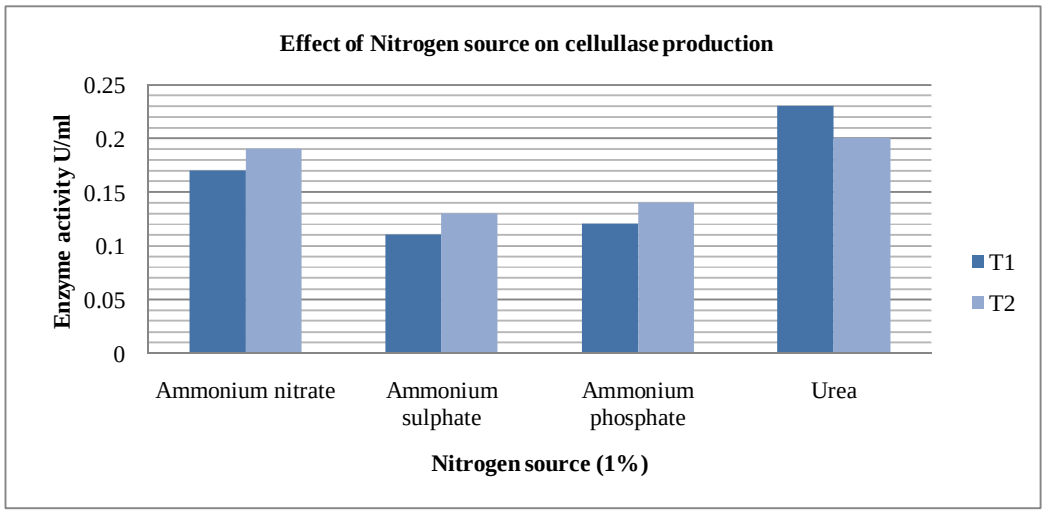

Figure 4. Effect of Nitrogen source on cellulase production by T1 and T2

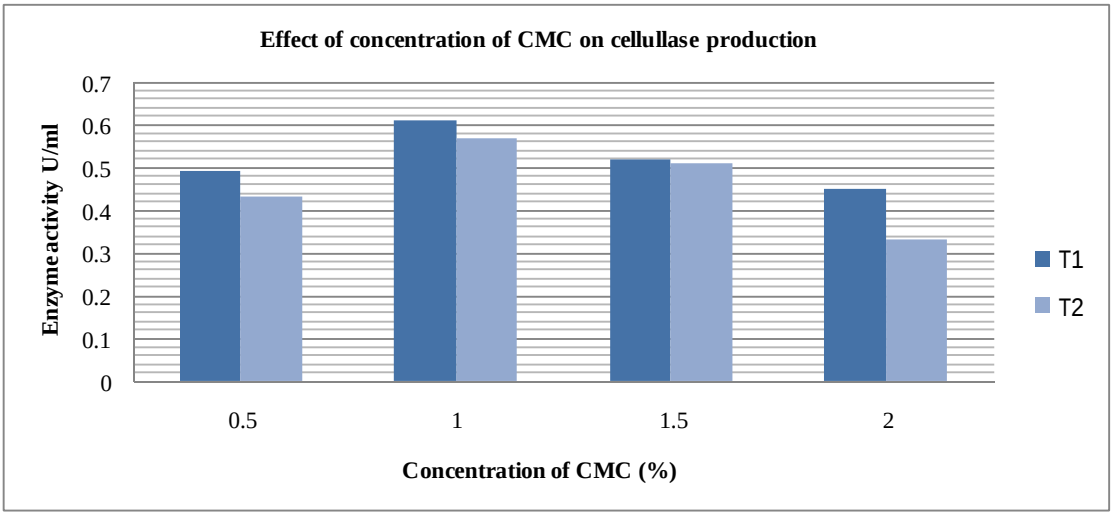

Figure 5. Effect of Concentration of CMC on cellulase production by T1 and T2

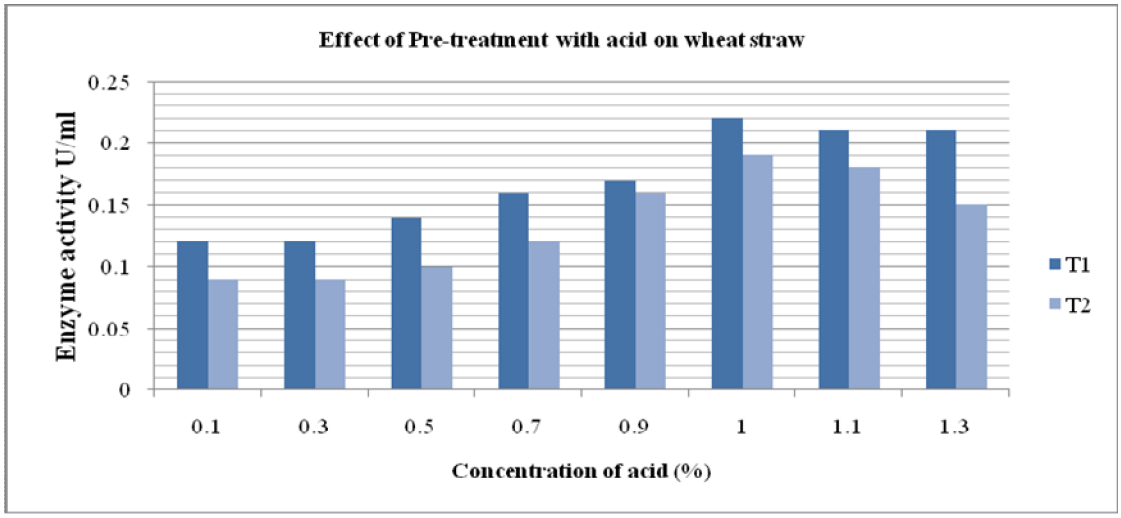

Figure 6. Effect of Pre-treatment with acid on wheat straw by T1 and T2

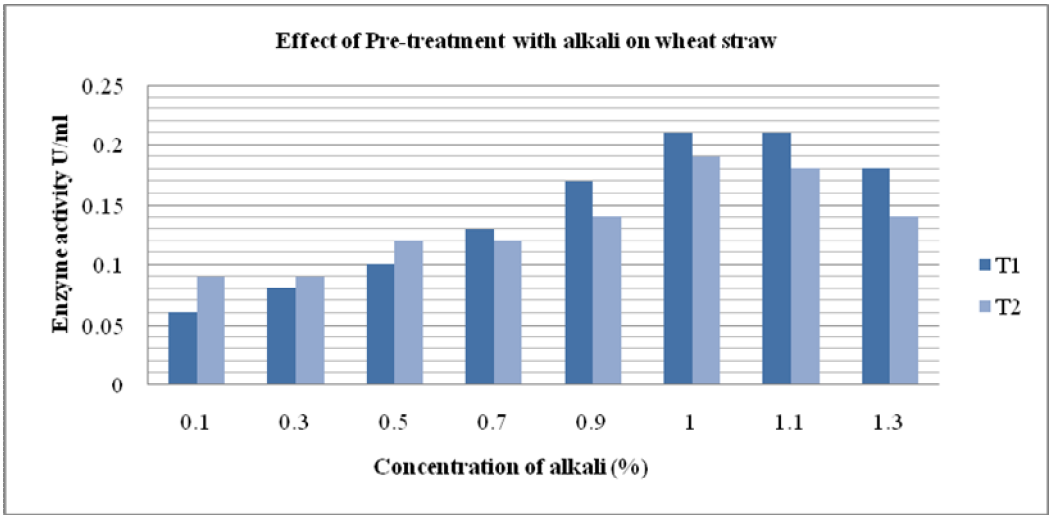

Figure 7. Effect of Pre-treatment with alkali on wheat straw by T1 and T2

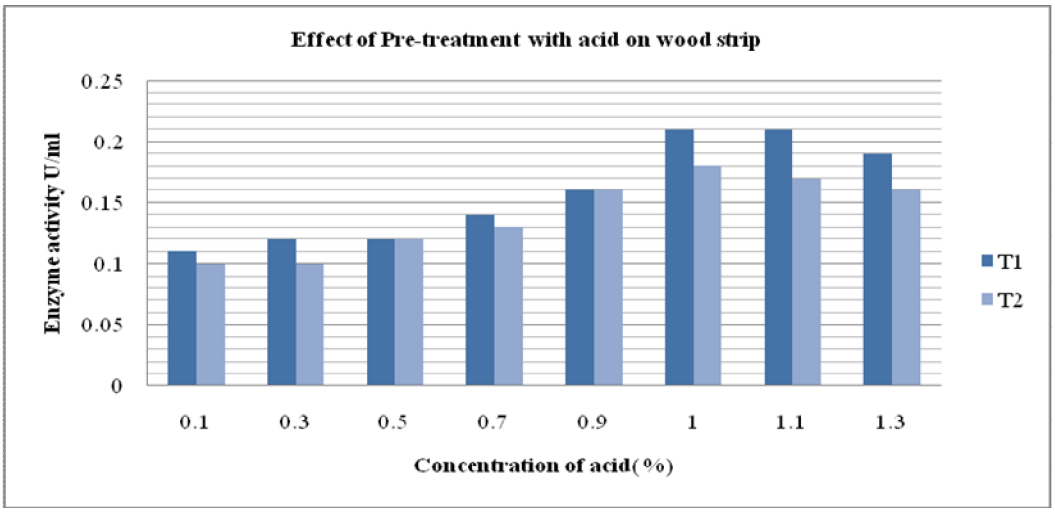

Figure 8. Effect of Pre-treatment with acid on wood strip by T1 and T2

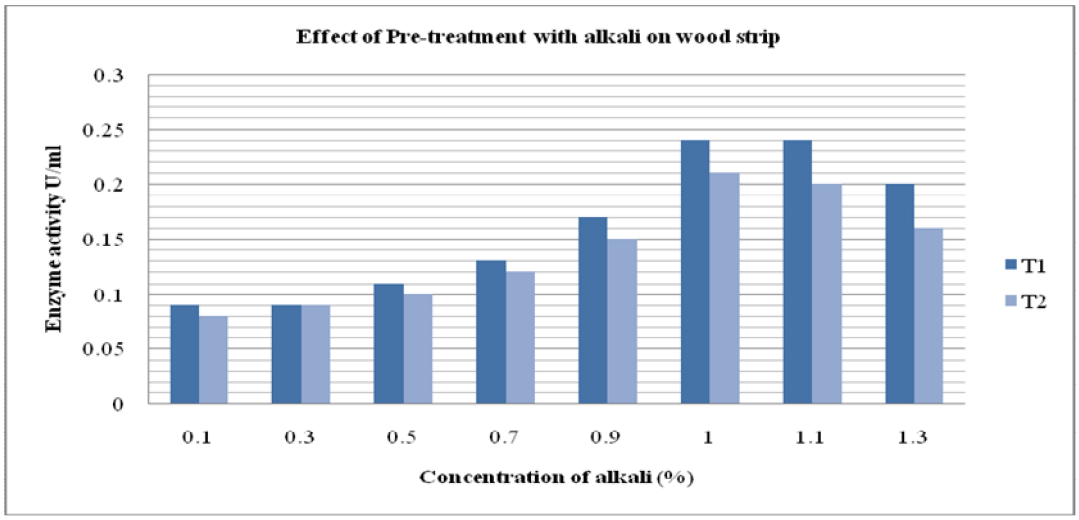

Figure 9. Effect of Pre-treatment with alkali on wood strip by T1 and T2

CONCLUSION

The potential cellulase producing bacterial strains T1 and T2 were isolated and selected for potential cellulase activity. These T1 and T2were characterized from their morphological, cultural and biochemical analysis and tentatively identified as *Bacillus sp*. Partial purification of cellulase was done and the enzyme activity and specific activity was determined. The optimum parameters had significant effect on cellulase activity. From the determination of enzyme activity and specific activity, T1 was appeared to be most efficient cellulase producer. As the cost of ethanol production is tightly associated with production of cellulase enzyme, the potential enzymatic degradation of cellulosic wastes by the thermophilic cellulase enzymes is a feasible alternative for the conversion of lignocelluloses into fermentable sugars and fuel ethanol**.** Further improvement in the performance of cellulase can be improved by mutagenesis and protein engineering techniques for the batter industrial applications.

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