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RESEARCH ARTICLE

Isolation and characterization of extracellular cellulase using *Bacilluls subtilis* from mangrove soil

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SUMMARY : A novel thermostable extracellular cellulases producing *Bacillus subtilis* was isolated from Mangrove soil. The bacteria were grown on carboxymethyl cellulose agar at 45°C and screened for the cellulase activity using Congo red method. The gram staining and biochemical tests had confirmed the microorganisms as *Bacillus subtilis*. Cellulose is commonly degraded by an enzyme called "cellulase". Cellulase is used for commercial food processing in coffee, textile industry and in laundry detergents. Maximum enzymatic activity was found at following optical parameters pH 7 (0.382 IU/ml) temperature 45°C (0.620 IU/ml); nitrogen source 0.6g (0.398 IU/ml) and incubation period 5 days. In addition, protein determination of different pH, temperatures and nitrogen sources was found to be most suitable for cellulase production.

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Key Words :
Mangrove soil, *Bacillus subtilis*, Carboxy methyl cellulose, Congo red

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Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis However, there are also cellulases produced by a few other types of organisms, such as some termites and the microbial intestinal symbionts of other termites. Several different kinds of cellulases are known, which differ structurally and mechanistically (Watanabe *et al.*, 1998). Cellulase enzyme hydrolyses a 1, 4 glycosidic bonds in cellulose polymer to release glucose units. Cellulase is produced by microorganisms (moulds, fungi, and bacteria) during their growth on cellulolysic material. Cellulolytic enzymes account for 20% of world's enzyme markets. Thermophilic bacterial cellulases have been frequently reported from *Bacillus* sp. (Ray *et al.*, 2007). Cellulose is commonly degraded by an enzyme called "cellulase" (Immanuel *et al.*, 2006).

In the recent years one of the most important biotechnological applications is the conversion of agriculture waste and all lignocellulosics into products of commercial interest such as ethanol, glucose and single cell products (Ojumu *et al.*, 2003). Cellulases refer to a family of enzymes which act in concert to hydrolyze cellulose. Cellulase is used extensively in the textile and food industries, bioconversion of lingocellulosics waste to alcohol, animal feed industry as additive, isolation of plant protoplast in plant virus studies metabolic investigation and genetic modification experiments (Bhat, 2000).

Celluloses are the biggest component and can be transformed into energy sources, paper, single-cell protein, glucose, and sorbitol (Putarau, 1969; Coral *et al.*, 2002). One of the goals in biotechnological development is to open the way to utilize microbes in waste bioconversion. Microbes used to treat cellulose-containing wastes could produce extra-cellular enzymes that were able to degrade cellulose material into their smaller components (Bedford and Partridge, 2001).

EXPERIMENTAL METHODOLOGY

Sample collection :

The Mangrove soil samples were collected

from Muthupettai, Thiruvarur(Dt), Tamil Nadu, India.

Physico - chemical analysis of soil :

Physiological characteristics relate to temperature, pressure, pH and salinity of medium .The physical chemical parameters of soil sample were carried out by using soil analysis kit (model 191E). The soil temperature. pH, electrical conductivity, turbidity, salinity, total dissolved solids (TDS) and dissolved oxygen were determined.

Isolation of bacteria (Serial dilution and spread plate method):

A serial dilution is the stepwise dilution of a substance in solution usually the dilution factor at each step is constant, resulting in a geometric procession of the concentration in a logarithmic fashion. A tenfold dilution for each step is called a logarithmic dilution or log dilution. A tenfold dilution could be 1M, 0.1M, 0.01M and 0.001M and soon. Serial dilution is done to accurately produce highly diluted solutions resulting in concentration curves with a logarithmic scale. Because of the reduction in the number of the bacteria due to dilution, isolate is obtained.

Morphological analysis :

Cultural characterization **:**

The colony morphology was studied based on the colour, shape, size and margin of the colonies.

Screening for enzymes production :

For cellulose producing bacteria, the dilution pour plate or spread plate techniques employed using CMC agar media. The plates were incubated at 45, 50 or 55^{0}C for 24 hours. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% congo red for 15 min and washed with 1M Nacl (Apun *et al.,* 2000). To indicate the cellulose activity of the organisms, diameters of clear zone around colonies on CMC agar were measured. Besides, a more quantitative assay method was used to determine the cellulose activity of the selected bacterial isolate in liquid medium. The cellulose activity of each culture was measured by determining the amount of reducing sugars liberated by using a dinitrosalicylic acid (DNS) method (Miller, 1959). A bacterial isolate with the highest cellulose activity was selected for optimization of cellulose production.

Cellulase assay method :

Procedure **:**

Cellulose activity was measured by a DNS method, through determination of reducing sugars liberated (Krootdilaganadh, 2000). 0.5 ml of 0.05 M citrate buffer pH 4.8 were incubated 30 minutes at 50^{o}C before adding 2 ml of DNS solution. The treated samples were boiled for 15 min prior to cool down in cold water for colour stabilization. The optical density was read at 540 nm against reagent blank by a spectrophotometer. OD is proportional to the concentration of the enzyme present. The more the enzyme activity the more the colour change.

Optimization of temperature for cellulase activity :

In order to determine the effect of temperature on cellulose production, the selected bacterial isolate was grown in CMC broth and incubated at 25, 35, 45, 55 and 65^{o}C for 5 days. Culture broths were then centrifuged at 140 rpm for 20 minutes to obtain supernatants which were later measured cellulase activity.

Optimization of pH for cellulase activity :

The effect of initial media pH on cellulose production was conditioned by adjusting the CMC broth to pH 4, 5, 6, 7 and 8 bacterial inoculation. After 5 days of incubation at 45^{o}C, culture broths were then centrifuged at 140 rpm for 20 minutes to obtain supernatants which were later measured the cellulose activity.

Optimization of nitrogen source for cellulase activity :

The appropriate nitrogen source (urea) for cellulase production by utilizing the *Bacillus subtilis*. The fermentation medium was supplemented with organic compound at different concentrations such as 0.2, 0.4, 0.6, 0.8 and 1.0g level, replacing the prescribed nitrogen source of the fermentation medium.

EXPERIMENTAL FINDINGS AND DISCUSSION

Five different types of bacterial cultures such as *Micrococcus luteus, Salinococcus reseus, Brevibacterium linens, Saccharococcus thermopholes* and *Bacillus subtilis* were isolated from Mangrove soil from Muthuppettai, Thiruvarur (Dt). A locally isolated bacterium, successfully screened for cellulase production was identified as *Bacillus subtilis*.

Physically the texture of all Mangrove soil sample was sandy. The physico-chemical parameters were such as pH (7.2), electrical conductivity (0.48 ms/cm), temperature (34.9.), salinity (16.2 ppt), turbidity (29 NTU). Available nitrogen (kg/ac) (83.7), total dissolved oxygen (42.1) mg/ l and total dissolved solid 0.19ppt were recovered from the soil samples of costal area, Muthupattai, Thiruvarur district (Table 1).

Table 1 : Physico-chemical analysis of soil sample

Sr. No.	Physico-chemical parameters	Mangrove soil
1.	Soil texture	Sandy
2.	pH	7.2
3.	Temperature (ºC)	34.9
4.	Electrical conductivity (ms/cm)	0.48
5.	Salinity (ppt)	16.2
6.	Turbidity (NTU)	29
7.	Total dissolved solids (ppt)	0.19
8.	Dissolved oxygen (mg/litre)	42.1
9.	Available nitrogen (kg/ac)	83.7

Bacterial species from Mangrove soils were isolated by serial dilution method plated in Nutrient agar medium. Then the plates were incubated at 37°C for 24-48 hours in an inverted position. Bacterial cultures were maintained in Nutrient broth, and as well as Nutrient slant for further studies.

Five different types of bacterial cultures were isolated from the master plate. Then these cultures were subjected to biochemical test for identification. The results are given in (Table 2). Gram staining of the cultures proved five strains. Among the 5 isolates, one strain showed higher enzyme activity when compared to other strains. Only one of these cultures was gram positive rod and another four strains were Gram positive cocci isolated from Mangrove soil.

The characteristics of *Balillus* species, cultivated on Nutrient agar medium was that the clear zone of *Bacillus* species was wider than that on CMC (Carboxyl methyl cellulase), medium (37.4 mm vs 15.60 mm) (Dharma, 1998). The effect of the pH on the crude cellulose activity of *Bacillus subtilis* was examined at various pH ranging from 4.0 to 8.0 as shown in Fig. 1. The enzyme has a broad range of pH activity (pH 6-8) with optimal at 7 which was close to the optimal pH value of most bacterial cellulases.

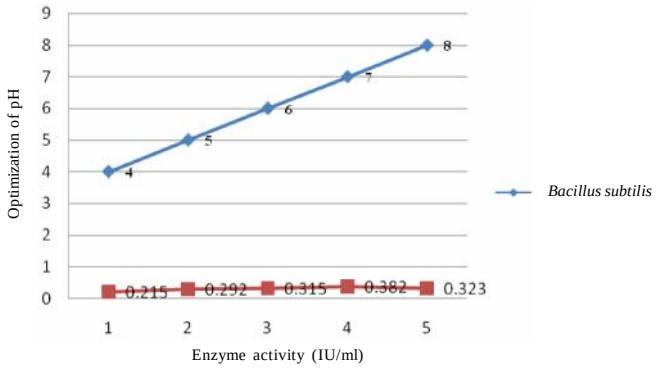

Fig. 1 : Effect of pH on cellulase production from *Bacillus subtilis*

Lee *et al*. (2002) suggested that the CMCase (Carboxyl methyl cellulase), exhibits a pH optimum of approximately 4, while the pH optimum of β-glucosidase was between pH 5 and 6.

The effect of temperature on crude cellulase was determined at various temperatures ranging from 25°C to 65°C. The enzyme showed a good activity (0.620 U/ ml) at 45°C, the optimum temperature (Fig. 2).

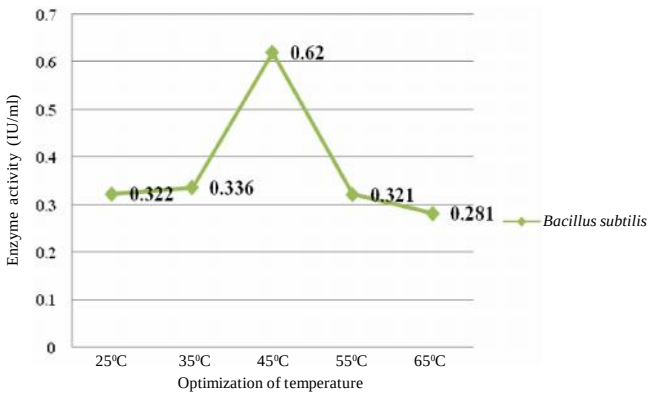

Fig. 2 : Effect of temperature on cellulase production from *Bacillus subtilis*

The alkaline celluloses from *Bacillus* species revealed an optimum from *Bacillus* 40 °C to 60°C (Christakopoulos *et al.*, 1999).

To evaluate the effect of nitrogen source on cellulase formation, the nitrogen source in the CMCase medium was replaced by nitrogen source at a different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0g). Data revealed that the supplementation of organic nitrogen source stimulated the cellulase yield and activity. The maximum enzyme activities were obtained with nitrogen source (0.6g) which brought about on improvement in the concentrations of 0.398IU/ml (Fig. 3). In the present

Table 2 : Morphological and biochemical characterization of bacterial cultures

Sr. No.	Test	B1	B2	B3	B4	B5
1.	Gram staining	+ve cocci	+ve cocci	+ve cocci	+ve cocci	+ve rod
2.	Motility	Non-motile	Non-motile	Non-motile	Non-motile	Motile
3.	Indole	–ve	–ve	–ve	–ve	+ve
4.	MR	–ve	–ve	+ve	+ve	–ve
5.	VP	+ve	–ve	–ve	–ve	+ve
6.	Citrate	+ve	+ve	+ve	+ve	+ve
7.	Catolase	+ve	+ve	+ve	+ve	+ve
8.	TSI	+ve	+ve	+ve	+ve	+ve
9.	Urease	+ve	+ve	+ve	+ve	+ve
10	Glucose	+ve	+ve	+ve	+ve	+ve
11.	Oxidase	+ve	+ve	+ve	+ve	+ve
12.	Bacterial cultures name	*Micrococcus luteus*	*Salinococcus reseus*	*Brevibacterium linens*	*Saccharococcus thermopholes*	*Bacillus subtilis*

+ Positive, –Negative

study, the organic nitrogen source was found to be more suitable for optimizing cellulose production by *Bacillus subtilis*.

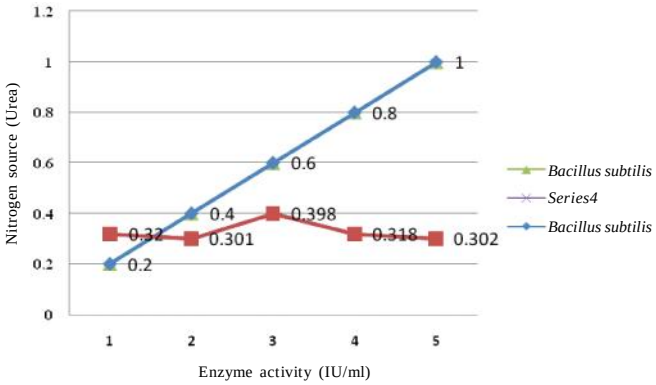

Fig. 3 : Utilization of nitrogen source from *Bacillus subtilis*

The value of the cellulose activity of both *Bacillus* produced by *Bacillus subtilis* cultivated for 72 hr on the medium completed with nitrogen source have been many efforts to generate *Bacillus subtilis* with high ability to produce cellulose than can degrade cellulose. A higher production of cellulose when CMC served as substrate may be as a result of induction of the enzyme, since cellulose is known to be a universal inducer of cellulose synthesis (Chundakkadu, 1999). The high amount of protein produced of *Bacillus subtilis* in all pH and temperature values are presented with Fig. 4-6. In the present study, the maximum pH, temperature and nitrogen source (urea) was observed in 6.0 (0.398 IU/ml), 25ºC (0.450 IU/ml) and 1.0 (0.352 IU/ml) cellobiase activity was shown by the isolate (*Bacillus subtilis*).

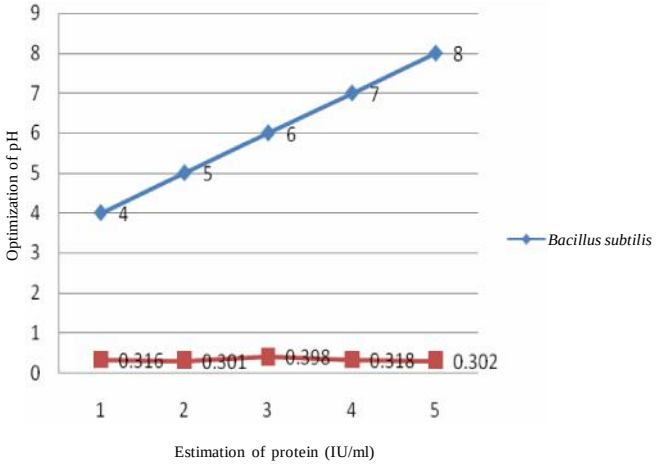

Fig. 4 : Estimation of protein in different pH of cellulase production

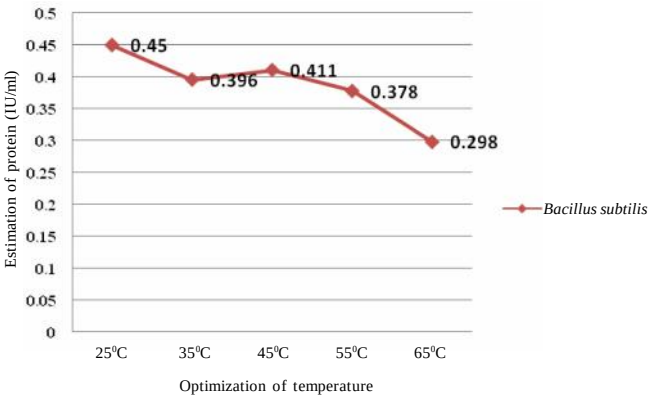

Fig. 5 : Estimation of protein in different temperatures of cellulase enzyme production

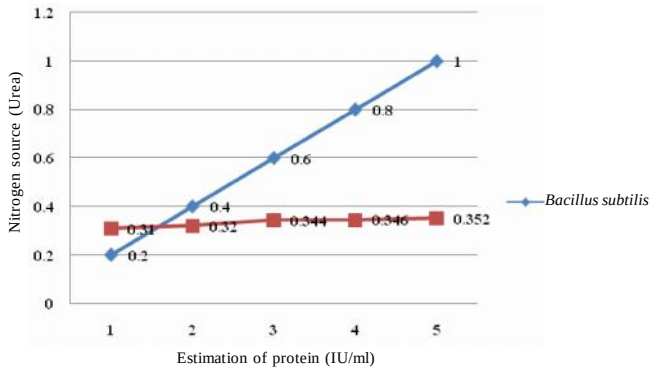

Fig. 6 : Estimation of protein in nitrogen source (urea) of cellulase enzyme production

Stephen *et al.* (2003) reported that this method is a combined assay for endo-and exo-glucanases invetro. Results revealed that incubation at pH value 7.00 was best where the recorded cellulose activity was 89.00 UmL^{-1} and the protein concentration and the biomass yield were 0.95 and 9.30 mg mL^{-1} and also the incubation at temperature 35ºC was best where the recorded cellulose activity was 95.00 UmL^{-1} and the protein concentration and the biomass yield were 1.10 and 9.00 mL^{-1}, respectively.

Conclusion :

Five different types of bacterial cultures such as *Micrococcus luteus, Salinococcus reseus, Brevibacterium linens, Saccharococcus thermopholes* and *Bacillus subtilis* were isolated from Mangrove soil from Muthuppettai, Thiruvarur (Dt). A locally isolated bacterium was successfully screened for cellulase production and was identified as *Bacillus subtilis.* The organic nitrogen source (urea) was found to be more suitable for optimizing cellulase production by *Bacillus subtilis.* The optimum pH for the maximum growth of organism was 7 at 45ºC. There was a maximum retention

of growth over a period. The optimum temperature for the activity of cellulase from *Bacillus subtilis* was found to be 45ºC.The highest amount of protein produced for *Bacillus subtilis* in all pH, temperature and nitrogen source. Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present.Cellulase is used as a treatment for phytobezoars, a form of cellulose bezoars found in the human stomach.

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