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Screening and characterization of cellulase producing bacteria from soil and waste (molasses) of sugar industry

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Abstract

The aim of present work is to illustrate the screening and characterization of cellulytic bacteria from soil and waste (molasses) of sugar industry. Soil and waste samples (molasses) from Colony sugar mills Phalia, Punjab, Pakistan were used for the screening of cellulytic bacteria by serial dilution and pore plate method. Bacteria were further Characterized by morphological and biochemical tests. Submerged fermentation process was used for enzyme production. Different production parameters: temperature, pH, incubation time and substrate concentration were optimized. Soluble proteins in the culture supernatant of isolated bacteria were measured by the dye binding method of Bradford. Enzyme activity was measured by dinitrosilsalic acid (DNS) method.Out of 26 isolates six were selected on the basis of clear zone produced 7mm ≥. These six potential isolates were further screened for cellulytic activity among which one SM3-M8 exhibited promising activity of cellulase. This bacterial isolate was then characterized by morphological and biochemical tests and identified as *Bacillus sp.* SM3-M8 gave maximum cellulase production and activity at temperature 45°C, pH 7, CMC concentration 0.5% after 48 hours of incubation. Sugar industrial wastes provided a good source for isolation of cellulase producing bacteria. Isolation and screening and characterization of microbes for cellulase production provided a valuable and novel enzymes for the conversion of lingocellulosic waste into ethanol.

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Introduction

Lignocellulose is one of the most abundant biopolymers in nature. With the scarcity of fossil fuels there is increase need to find substitute sources for renewable energy and fuels. To fulfill this need, there is a continuous interest in the bioconversion of lignocellulosic biomass to reducing sugars using cellulose degrading enzymes (Cellulases) (Singhania *et al.,* 2010). Cellulose is the chief component of plant biomass (Camassola *et al.,* 2007). Plants produce 4×10^9 tons of cellulose per year (Coughlan *et al.,* 1990). It is a polymer of ß-1, 4 linked glucose units. Its crystalline structure and insoluble nature create a huge challenge for enzymatic hydrolysis. There are many sources to get lignocellulosic biomass and produce cellulose. These include municipal waste, agricultural residues, forestry or pulp, paper excesses, energy crops, sugar wastes and Switchgrass (Greene *et al.,* 2004). The use of cellulose as a renewable source of energy has made cellulose hydrolysis, the subject of current research for industrial applications (Bhat *et al.,* 2000).

Cellulases are the potential enzyme system that can degrade and hydrolyze lignocellulosic and cellulosic waste. It is a class of enzymes that break cellulose into glucose monomers (Cai *et al.,* 1999). Bacterial and fungal cellulases are traditionally classified into three classes: Endoglucanases (EGs) (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and Carboxymethyl cellulase (β-glucosidases) (EC 3.2.1.21) (Kim *et al.,* 2008) depending on their ability to degrade carboxymethylated cellulose (CMC), whereas EGs being the most efficient (Henriksson *et al.,* 1999). Cellulase enzyme has its importance due to key role in industrial applications (Bhat *et al.,* 2000). It is used for bioremediation (Zahangir *et al.,* 2005), in food processing (Chandara *et al.,* 2005), in paper, pulp industry, as a supplement in animal feed industry (Bhat *et al.,* 2000; Chandara *et al.,* 2005) and in textile industry (Ali *et al.,* 2008).

Researchers keep on working to isolate microorganisms with higher cellulase activity (Ray *et al.,* 2007). Microorganisms are important in conversion of lignocelluloses wastes into important products like biofule which is produced by fermentation (Lynd *et al.,* 2002). Bacteria which have fast growth rate as compared to fungi can be used for cellulase production. The potential cellulase producing bacteria are *Cellulomonas*, *Pseudomonas, Thermoactinomycetes* and *Bacillus* spp. (Godana *et al.,* 2007). Complete and successful bioconversion of cellulosic materials largely depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes (Alam *et al.,* 2004). For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more useful cellulases from variety of sources such as soil (agricultural, industrial or municipal), decayed plant materials, hot springs, organic matters, feces of ruminants and composts (Doi *et al.,* 2011) but only a few of them produces considerably large quantities of bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro*. The key stage in the development of an industrial fermentation process is to isolate strains capable of producing the target product in commercial yields. This approach results in intensive screening programs to test a large number of strains to identify high producers having novel properties.

The main objective of this study was to isolated novel bacterial strains with putative cellulase activity from sugar industry wastes such as molasses and soil. Molasses are cellulosic in nature hence the microbial population present in soil and waste (molasses) should has the capacity to degrade cellulose because they have to produce cellulases (CMCase) to hydrolyze that material (cellulose present in waste) for their growth. Five species of bacteria, four of yeast and six species of fungi were also reported from molasses (Shanker *et al.,* 2011). So, the present research was planed to screen the bacterial population present in sugar wastes of Colony sugar Mill, Phalia. Pakistan and also identify the potent strains for cellulase production. Furthermore, we also screened the potential strain among these bacterial strains that gives maximum cellulase production.

Different production parameters were also optimized for enhanced cellulase production.

Material and methods

Sample collection

The soil samples (10gm) and molasses samples (10gm) were collected from Colony Sugar Mill Phalia, Punjab, Pakistan. Soil samples were collected from surface and 5, 10 and 15 cm depth with the help of sterile spatula. Samples were collected in sterile polythene plastic bags and labeled properly. Three soil samples and three molasses samples were collected. Samples were stored at 4°C before further use. Screening and isolation was performed in the lab of Dept. of Biochemistry and Molecular Biology University of Gujrat, Gujrat, Punjab, Pakistan. Soil samples were labeled as: SM1-S, SM2-S and SM3-S and molasses samples were labeled as: SM1-M, SM2-M and SM-3M.

Enrichment of bacterial population and isolation of cellulase producing bacteria

Cellulase producing bacteria were isolated from soil and molasses by using culture enrichment and pore plate methods. The Modified Han's (MH) medium along with carboximethyl cellulose as selective carbon source was used for this purpose (Kasana *et al.,* 2008).

Screening of cellulase producing bacteria

Primary screening of colonies was made by the mwthod of congo red (Ariffin *et al.,* 2006). Formation of clear zone around colony indicates the activity of cellulase enzyme produced by bacteria of that colony of respective sample either soil or molasses (Kaur *et al.,* 2012). The diameter of hydrolysis zone indicates the best cellulase producer bacterial colony. These colonies were further selected for cellulase production and secondary screening (Immanuel *et al.,* 2006).

Cellulase enzyme production and activity assay

Potential isolates obtained were then observed for cellulase production. For cellulase production we use submerged fermentation process (Irfan *et al.,* 2012). Enzyme activity was measured by using Dinitosacialic Acid (DNS) method (Miller *et al.,* 1959). The activity was measured as IU/ml which indicates the amount of CMCase necessary to release 1µmole of reducing sugar per ml per minute at 60°C and pH9 (Shaikh *et al.,* 2013). Soluble proteins in the culture supernatant of isolated colony were measured by the dye binding method of Bradford (Bradford *et al.,* 1976).

Identification of selected strain

The strain selected on the basis of maximum enzyme activity was identified with the help of morphological and biochemical tests. In morphological tests: grame staining, colony morphology, shape of bacteria and motility tests we performed (Apun *et al.,* 2000). In Case of biochemical identification: Indole test, methyl red test, citrate utilization test, catalase test, oxidase test, nitrate reduction test, amylase, carbohydrate (Glucose, Fructose, Sucrose, Sorbitol) fermentation test by standards methods (Buchanan *et al.,* 1974).

Optimization of different parameters for cellulase production

The parameters like temperature, pH, Substrate Concentration, and incubation time was optimized for selected bacterial strain. For the optimization of temperature fermentation process was carried out at temperature range of 30°, 35°, 37°, 40° & 45°C. To observe the effect of substrate concentration on cellulase production, we prepare fermentation media containing CMC concentration 0.2%, 0.4%, 0.5%, 0.8% & 1%. Different values of pH ranged from 5 to 8 are selected for studying their effect on the production of cellulase. After the preparation of media the pH was adjusted with the help of pH meter. Sodium hydroxide and Hydrochloric Acid was added to adjust the pH at required value. To attain the maximum cellulase production fermentation was carried out at different incubation times ranged from 24 to 120 hours.

Results and discussion

Isolation and screening of cellulase producing bacteria

Bacteria are well known agents of decomposition of organic matter in general and of cellulosic substrate

in particular as reported by (Lynd *et al.,* 2002). We know that bacteria utilize wide range of cellulosic waste. Therefore interest in the isolation and screening for novel cellulase producing bacteria was increased. Present study was carried with an aim of isolating, screening and identification of efficient cellulase producing bacteria from soil and waste (molasses) of sugar industry.

Table 1. Zone of clearance of cellulase enzyme produced by bacterial isolates isolated form soil and molasses.

Sr.No.	Sample Name	Diameter of clear zone (mm)
1	SM1-S (2)	7.5
2	SM1-S (4)	8.5
3	SM1-S (8)	7
4	SM2-S (1)	7.5
5	SM2-M (3)	7
6	SM3-M (4)	7
7	SM3-M (5)	8
8	SM3-M (6)	7.5
9	SM3-M (8)	9.5

CMC agar was a selective media for the growth of cellulolytic bacteria because cellulase producing bacteria can only hydrolyze cellulose as carbon source (Rahna *et al.,* 2011). The screening of cellulase producing bacteria were performed (Kaur *et al.,* 2012). There are around 26 isolates (bacteria) were grown on CMC agar of total 6 samples. Among these 26 isolates 17 were eliminated on the basis of similar colony and diameter less than 7mm. SM3-M (8) has highest clearing zone of 9.5mm among 9 isolates which were considered for further studies (Table 1). The zone produced by all isolates was shown in figure 1. Three potential isolates of soil and molasses were considered for secondary screening.

Table 2. Enzyme activity, specific enzyme activity and protein concentration of different isolates of soil and molasses samples.

Sr.No.	Sample Name	Enzyme Activity (IU/ml)	Protein Concentration (mg/ml)	Specific Enzyme (IU/mg)
1	SM1-S (2)	1.437	0.968	1.484
2	SM2-S (1)	2.742	0.965	2.841
3	SM1-S (4)	2.030	1.010	3.0
4	SM3-M (5)	2.680	0.912	2.938
5	SM3-M (6)	2.290	0.827	2.768
6	SM3-M (8)	3. 198	1.117	3.042

Secondary Screening and cellulase enzyme production assay

On the basis of primary screening the 6 potential isolates were then observed for their enzyme productivity in liquid fermentation process. For the measurement of enzyme activity of crude enzyme samples the filtrate of the fermented solution was assayed according to cellulases enzyme assay method (Shaikh *et al.,* 2013).

Enzyme activity assay

Enzyme activity, protein concentration and specific activity of samples were given in table 2. SM3-M (8) has maximum activity as well as protein concentration. The isolate SM3-M (8) was then selected for further study of morphological and biochemical identification.

Table 2, showed that all these isolates of soil and molasses have a potential to hydrolyze cellulose with

the help of cellulase produced by them in fermentation process. But SM3-M (8) has maximum potential to grow in such type of waste and produce a significant amount of cellulase which can degrade lignocellulosic waste. It was identified on the basis of different morphological and biochemical test (Rahna *et al.,* 2011).

Table 3. Staining and Biochemical characteristics of the bacterial isolate SM-3M (8).

Sr.No.	Morphological or Biochemical Test	Result
1	Gram staining	+
2	Motility test	+
3	Indole	-
4	Methyl red	-
5	Citrate utilization test	+
6	Catalase test	+
8	Amylase test	+
9	Nitrate reduction test	+
	Carbohydrate fermentation	
10	Glucose	+
11	Fructose	+
12	Sucrose	+
13	Sorbitol	+

*Negative & + Positive.

Morphological and biochemical identification

The isolate SM-3M (8) was purified and single colony was achieved by repeated sub-culturing on the MH agar medium supplemented with CMC at regular intervals of time and then stored at 4°C.The isolate was identified and species name was assigned on the basis of morphological and biochemical characteristics which was given in table 3 (Irfan *et al.,* 2012). The morphology of isolate SM3-M (8) is light creamy brown, has flat rough colonies with irregular edges. The isolated microbes were motile and spread rapidly on the surface of CMC agar medium. They were grame positive bacilli. The isolate SM3-M (8) was oxidase positive, glucose, fructose, sucrose, sorbitol fermenting, indole and methyl red negative, Citrate Catalase, Amylase and Nitrate reduction tests were positive which are biochemical properties of *Bacillus sp*. Isolate SM-3s (8) is identified as *Bacillus sp*. Similar results were also reported by Rahna *et al.,* 2011; Shanmugapriya *et al.,* 2012.

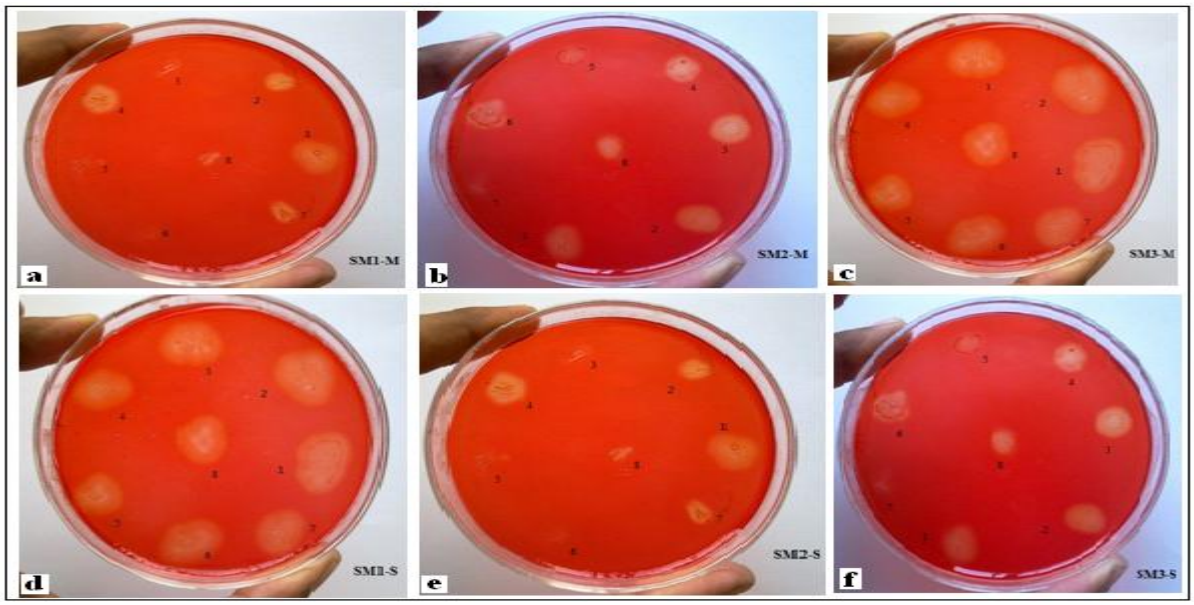

Fig. 1. Clearing Zone Produced by the Isolates of Molasses (a, b & c) and Soil (d, e & f) Samples.

Optimization of different production parameters for cellulase enzyme activity

Optimum parameters were determined for cellulase production and activity from the efficient isolate SM3-M according to (8). After fermentation at the different parameters the crude enzyme product was collected for determination of enzyme activity.

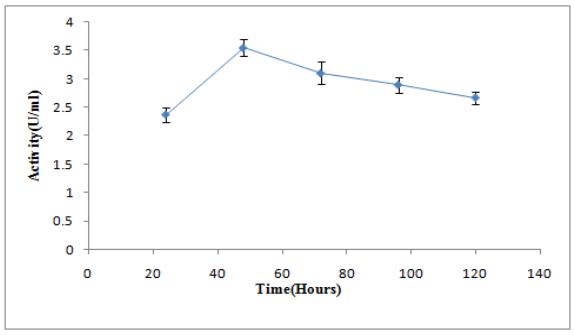

Fig. 2. Effect of Incubation Time on Production.

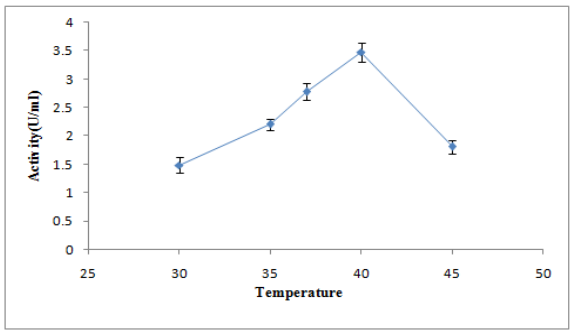

Fig. 3. Effect of Temperature on Enzyme Production.

Effect of incubation time on cellulase activity

The effect of incubation period for enzyme production by isolate SM-3M (8) was observed in the production medium MH and CMC in that act as a substrate for enzyme production. The activity was observed from 24 hours to 120 hours. The activity determined ranged from 2.371 IU/ml to maximum 3.547 IU/ml was also shown in Figure 2. The maximum activity or production of enzyme was found between 48 and 72 hours. The major peak of activity was observed at 48 hours which was 3.547 IU/ml. Same Optimum incubation time for maximum cellulase production by *Bacillus sp.* was reported by Verma *et al.* 2012**.** It may be due to decrease in nutrients of media and respective cell death in the medium (Ariffin *et al.,* 2006). After this the time of incubation was kept 48 hours and other parameters temperature, pH, Substrate concentration was changed respectively to get high yield and activity of enzyme.

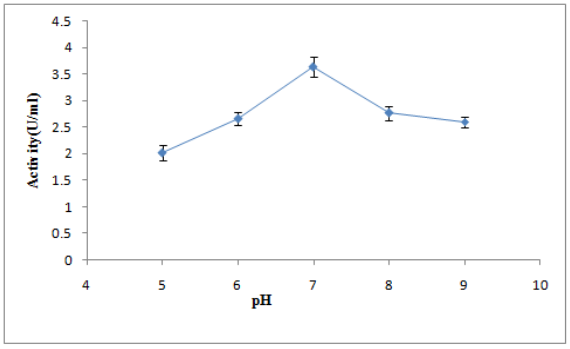

Fig. 4. Effect of pH on Enzyme Production.

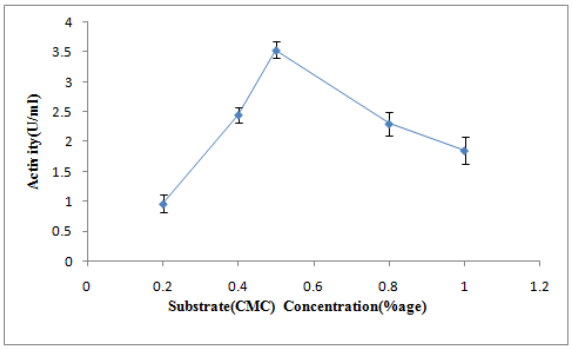

Fig. 5. Effect of Substrate Concentration.

Effect of temperature on cellulose activity

Temperature plays an important role in enzyme production. Effect of temperature from 30°C to 45° C was studied. Enzyme activity was found in the range of 1.485 IU/ml to 3.479 IU/ml. Maximum activity of enzyme produced by SM3-M (8) (*Bacillus sp.*) was at 40° C followed by 37° C was shown in Figure 3 (Sethi *et al.,* 2013). Results indicate a gradual increase of enzymatic activity till 40° C and then sudden decrease at 45° C because at this temperature no growth was observed hence no activity was found. Many researchers' reported different temperatures for maximum cellulase production either in lab or at industrial scale using *Bacillus sp*. suggested that the optimal temperature for cellulase production depends upon the strain variation of microorganisms **(**Immanuel *et al.,* 2006). Maximum activity in case of *Cellulomonas and Bacillus sp*. was found at 40° C.

Effect of pH on enzyme activity

A study of the effect of initial pH of culture medium on cellulase production at 40°C showed that the pH of the medium had a slight effect on cellulase

production. Maximum growth and enzyme activity was observed at pH 7.0 which is 3.642 IU/ml. Similar observations was observed by Shaikh *et al.,* 2013 when he isolated cellulase producing bacteria from different environment. The enzyme activity at different pH values were shown in Figure 4. The cellulase activity in culture supernatant (Crude enzyme) increased from 2.017to 3.642 IU/ml with an increase of the pH of culture medium from pH 5.0 to 7.0. There was a decrease in cellulase activity from 3.642 to 2.6 IU/ml on increasing the pH from 7 to 9. Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. In general, higher cellulase activity was produced when *Bacillus subtilis* sp. was grown in the culture medium of neutral pH 7 (Verma *et al.,* 2012).

Effect of substrate concentration

Cellulase production by *Bacillus subtilis* sp. was studied by testing cellulase secretion in the culture medium using different substrate (CMC) concentrations at 40°C. CMC is used as a substrate for cellulase production due to its less complexity and easy digestion by the microbes (Shanmugapriya *et al.,* 2012). Highest level of Cellulase activity and protein 3.534 IU/ml and 1.117 mg/ml respectively were produced when culture was grown on MH along with CMC at 0.5% for 48 hours at 40°C**.** CMC concentration ranged from 0.2 % to 1.5 % used and got maximum activity by *Bacillus subtilis* at CMC concentration of 0.5% (Shaikh *et al.,* 2013). The activity at different concentrations from 0.2% to 1% was shown in Figure 5.

Conclusion

With a view to develop an economically feasible technology, we focused our research efforts mainly on screening from natural habitat for microbe isolation that produces substantial amount of cellulases. Cellulase producing bacteria were isolated from soil and molasses of a sugar industry. Initially 26 isolates were primarily screened by staining the colonies of these isolates with Congo Red. Six potential isolate which produce clearwing zone 7.5 mm ≥ was selected for secondary screening (Cellulase production). Among these isolate SM3-M (8) of molasses sample shows maximum enzyme activity. SM3-M (8) produce clearing zone of 9.5 mm and give maximum activity 3.198 IU/ml. Which was further identified as *Bacillus sp* on the basis of morphological and biochemical characteristics. Different production parameters like temperature (40°C), time of incubation (48 hours), pH (7.0) and substrate concentration (0.5%) were determined.

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Competing interests

Authors have declared that no competing interests exist.

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