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ORIGINAL RESEARCH ARTICLE

Isolation of *Bacillus* spp. Bacteria from Soil for Production of Cellulase

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Abstract

Cellualse is one of the most important enzymes used in textile, detergent, paper, food and feed industries. Therefore, a study was undertaken to isolate *Bacillus* bacteria having the potential to produce cellulase from soil samples. 24 soil samples were analyzed and 54 presumptive *Bacillus* isolates were isolated after heating the soil samples at 80°C for 10 min. Among them 45 isolates showed enzyme activity ranging from 0.003 to 0.17 U/ml in test tubes containing 5 ml medium composed of (g/L) glucose 0.5 gm, peptone 0.75 gm, $FeSO_4$ 0.01 gm, KH_2PO_4 0.5 gm, and $MgSO_4$ 0.5 gm at 120 rpm, 37 °C and pH 7. Among them 1RW, 2WS, 3YR, 4WT, 6 RR, and 9SS showed 0.17, 0.15, 0.14, 0.15, 0.147 and 0.14U/ml enzyme activities, respectively. Production of cellulase by these isolates was further scaled up to shake culture containing 50 ml medium similar to that used in test tube culture. Among the isolates 1 RW showed the maximum activity. This 1 RW was identified by API kit and showed that 59 % belongs to *Bacillus licheniformis* strain (51% confirmation) or *Bacillus subtilis* (31% confirmation). Further gene analysis is required to confirm the species. The genetic improvement study will make the isolate a good source of cellulase.

Keywords: Cellulase, *Bacillus*, isolation, API kit, shake culter.

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Introduction:

Microbial cellulases have applications in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture [1]. In food industry, cellulases have an important application for extraction and also clarification of juices from fruits and vegetables [2]. It is also used in the conversion of cellulosic wastes into glucose [3] and the enzymatic saccharification of lignocellulosic materials for bio-fuel production [1]. Cellulases are successfully used in textile industries for finishing of cellulose-based textiles in wet processing; in paper industries for deinking of paper wastes and in animal feed industries for pretreatment of silage and grain to increase its nutritional value [1].

Cellulase can be produced by various fungal species such as *Aspergillus, Rhizopus, Trichoderma, Fusarium, Neurospora, Penicillium*, etc. [4]. Production of cellulases by some bacteria such as *Cellulomonas, Cellvibrio, Pseudomonas* sp, *Bacillus,* and *Micrococcus* has also been reported [5]. However, bacteria with comparatively higher growth rate can potentially be used in cellulase production. Nevertheless, production of cellulase by bacteria is not widely observed.

Sometimes bacteria are preferred for large scale production of enzyme because most of them produce large amount of thermostable extracellular enzymes, which are active at a wider pH range [6]. Since each microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties, new strains with higher activity and unique properties are always searched to meet the demands at a commercial level [7]. Among bacteria, the *Bacillus* strains are most important industrial enzyme producer as they have potential for production of large amount of extracellular enzymes [8].Therefore in this study an investigation was undertaken to isolate *Bacillus* bacteria capable of producing cellulase enzyme.

Materials and Methods

Screening and isolation of *Bacillus* spp.

Generally the *Bacillus* spp. are spore former. Therefore, the major interest was isolation of the spore forming rod shaped bacteria. For this purpose the 10 g soil sample was diluted in 90 ml sterile normal saline and heated at 80°C for 10 min to eliminate vegetative cells [9]. Cellulase-producing bacteria were isolated by the dilution spread plate method using CMC agar media in which CMC was the sole carbon source. The plates were incubated at 37°C for 48 hours. Gram staining of the bacteria was performed.

Selection of cellulolytic bacteria

The colonies found on the CMC agar plates were collected and a quantitative assay method was used to determine the cellulase activity of the selected bacterial isolates in liquid medium containing (g/L) glucose 0.5 gm, peptone 0.75 gm, $FeSO_4$ 0.01 gm, KH_2PO_4 0.5 gm, and $MgSO_4$ 0.5 gm in test tubes [5]. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated using a DNS method [10].

Bacterial identification

The assumed *Bacillus sp.* was identified using BioMerieux API 50 CHB/E Kit. Bacterial suspension was made with medium and each tube of the strip was then inoculated with the bacterial suspension. The bacteria fermented the carbohydrates to acids which decreased the pH and it was detected by the change in color of the indicator. The identification software identified the strain using the biochemical profile made up from the results. The colony selection, inoculum preparation, inoculation, incubation and interpretation of results were performed according to the manual provided by BioMerieux. The presumptive strain was determined by the software (apiweb TM) based on the results found by the API kit [11]. A positive test corresponding to acidification was indicated by the phenol red changing to yellow, except the esculin test (tube no. 25) where a change in color from red to black was observed as positive.

Enzyme production medium

Production medium contained (g/L) glucose 0.5 gm, peptone 0.75 gm, $FeSO_4$ 0.01 gm, KH_2PO_4 0.5 gm, and $MgSO_4$ 0.5 gm. 50 ml of media were taken in 100 mL conical flasks. The flasks were sterilized in an autoclave at 121°C for 15 min and after cooling, the flasks were inoculated with overnight grown bacterial cultures. The inoculated media were incubated at 37°C and 120 rpm in a shaker incubator for 48 h. After fermentation, the culture media were centrifuged at 6000 rpm for 10 min and the supernatant were used as enzymes.

Enzyme assay

Cellulase activity was measured following the method of Miller (1959) [10]. Briefly, a reaction mixture composed of 0.5 mL of crude enzyme solution plus 1.0 mL of 1% carboxymethyl cellulose (CMC) in Citrate buffer (pH 5.2) was incubated at 50°C in a shaking water bath for 30 min. The reaction was terminated by adding 3 mL of DNS reagent. The color was then developed by boiling the mixture for 15 min. OD of samples was measured at 540 nm against a blank containing all the reagents except the crude enzyme. One unit of cellulase is the amount of enzyme necessary to produce 1 µmol reducing sugar per min under the standard assay conditions.

Result and Discussion

For rapid screening of cellulolytic bacteria, agar media containing 0.5 % CMC as sole carbon source are flooded with congo red after incubation. Cellulolytic colonies can be detected by observing a surrounding pale orange to clear zone against red background. The cellulolytic bacteria can be screened directly on such plate, but replica plating (each colony is inoculated onto multiple plates) from master plate is recommended because flooded reagents interfere the isolation [12]. Another procedure reported where grams iodine was used instead of congo red. Since there is poor correlation between enzyme activity and size of halo the plate-screening methods using dyes are not quantitative [12]. In this study the colonies were directly selected and used for quantitative analysis of cellulase activity.

Bacillus spp. are moderately human friendly microorganisms. Various species are being used for the production of numerous industrial products such as enzymes [13], gamma polyglutamic acid [14], bacteriocin [15], biopesticides [16], waste management [17], probiotics [18] etc. One strain of *Bacillus* may produce different kinds of valuable products [19]. Therefore in this study the main target was the isolation of *Bacillus* spp. and the production of cellulase enzyme form the isolates. Since *Bacillus* is a spore former the isolation of this organism involved the heating of the samples at 80 °C for 10 min so that the vegetative cells were eliminated keeping the spores stable. Then gram positive rods were selected as presumptive

Bacillus. 24 soil samples were investigated. After the heat treatment the bacterial load was found to be lower. There were found in different types of colonies such as wrinkled large, smooth small and
smooth large colonies. All bacteria showed gram positive rod shaped results. Initially these organisms were grown in media in test tubes at 37 °C and 120 rpm in shaking incubators. The isolates produced very low amount of enzyme showing in the **Table 1**.

Table 1. Enzyme production by the presumptive *Bacillus* isolates.

Isolate	Enzyme activity (U/ml)	Isolates	Enzyme activity (U/ml)	Isolates	Enzyme activity (U/ml)	Isolate	Enzyme activity (U/ml)
1 RW	0.17	1 SL	0.074	2 WS	0.15	2 YR	0.124
3 YR	0.14	3 WS	0.11	4 WT	0.15	5 RW	0.012
5 SS	0.13	6 RR	0.147	6 WS	0.1	7 RW	0.026
7 SL	0.11	6 WS	0.1	7 RW	0.026	7 SL	0.11
7 SS	0	8 RW	0	8 SL	0.09	9 RW	0
9 SL	0	9 SS	0.14	10 SL	0.13	10 RW	0.016
10 SS	0.13	11 RW	0	11 SL	0	12 SL	0.135
12 RW	0.015	13 RW	0.002	14 SW	0	14 WW	0.018
15 WP	0.04	15 RW	0.05	15 SW	0.007	15 SS	0.11
16 RW	0	16 SW	0.035	17 RW	0.007	17 SW	0.035
17 SS	0.11	18 RW	0.004	18 SS	0.11	19 RW	0.0034
19 SL	0.018	20 RW	0.0013	21 RW	0.008	21 SS	0.01
22 RW	0.032	22 SS	0.04	22 SSp	0.045	23 RW	0.044
23 RS	0.055	24 RW	0.05	24 SS	0.043		

Figure 1. Biochemical test result of isolated *Bacillus* sp on API kit

Among the isolates 1RW, 3YR, 6RR, 9SS, 2WS and 4WT were selected for further experiment to find out whether these strains are stable at shake flask culture. In shake flask culture, the 1 RW showed the maximum activity among the isolates. This type of result (enzyme activity 0.5 to 0.9 U/ml) was reported for environmental strains such as *Bacillus* spp. [19-21] and *Cellulomonas* spp. [22]. In this study, the isolate 1RW was further identified by the API kit method. The results showed that the isolate belonged to the *Bacillus* genera and might be *Bacillus licheniformis*. The Figure 1 showed the biochemical reaction pattern of the isolate. Reaction 1, 4, 5, 6, 11, 12, 13, 17, 18, 19, 24, 25, 26, 27, 28, 31, 32, 35, 36, 37 became yellow which indicated positive results. Tube number 25 designated for esculine turned black in color.

Using the software it was determined that the isolate belonged to *Bacillus licheniformis* 51 % and *Bacillus subtilis* 31 %. The *B. licheniformis* and *B. subtilis* are genetically related [23]. These two strains are used to produce various microbial products. To remove the confusion further gene sequencing would be done. The natural strain normally produces a lower yield. Therefore further genetic improvement through mutation will be performed for 1RW. The bacteria are more suitable to produce enzyme than fungi because bacterial cellulases are more resistant to alkaline and thermophilic conditions and are good candidates for using in laundries [24]. Among the bacteria, the *Bacillus* spp. are more suitable because they have high growth rates, able to secrete enzymes in extracellular media and

generally regarded as safe [8]. To improve the enzyme production yield, optimization of medium and fermentation conditions as well as genetic improvement of natural strains, are required [25].

Conclusion

In this study, *Bacillus* sp. has been isolated and identified using API 50 CHB/E kit. This bacteria is capable of producing cellulase in submerge culture. The *Bacillus* isolate would be a good source of cellulase if further investigations in terms of medium optimization and genetic improvement are performed.

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