International Journal of Modern Agriculture, Volume 9, No.4, 2020
ISSN: 2305 - 7246

Screening, Characterization and Identification of Cellulase Positive Bacillus Spp. Isolated From Pond Sediment Samples

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Abstract

In the present investigation attempts have been made to isolate, identify and characterize the cellulase positive *Bacillus* species isolated from pond sediment samples. Out of 20 collected samples based on heat treatment survival and carboxymethyl cellulose interaction assay, 16 isolates were confirmed as *Bacillus* spp. owing cellulase activity. All the isolates were observed as Gram positive, endospore producing rod shaped aerobic bacteria in microscopic examination. Biochemical characterization of the isolates showed all the isolates to be positive for catalase, urease and oxidase activity, negative for indole and H_2S production, and variation in methyl red, Voges Proskauer, citrate and nitrate activity was observed. Carbohydrate utilization tests revealed all of the isolates to be positive for glucose, fructose, trehalose and cellobiose while most all strains were negative for melezitose, rhamnose and mellibiose. Extracellular enzyme activity production for caseinase and gelatinase was found be positive for all the isolates whereas variation in amylase, lecithinase and negative for lipase activity was observed with all the isolates. Physiological responses in relation to salt and temperature was found to tolerate maximum 7% of NaCl concentration and a temperature range of 60℃ for most of the isolates. All the isolates were identified as different strains of 3 major groups of *B. subtilis* (08), *B. pumilis* (02) and *B. cereus* (06). This study reveals that *Bacillus* spp. to be the dominant cellulase producers in pond sediment samples and can be employed in industrial production of cellulose degrading enzymes.

Key words: Bacillus spp., CMCase, Sediments, Biochemical tets

Introduction

Cellulose is one of the key structural resources and a carbonaceous constituent of aquatic plants. Since cellulosic materials are not functional in their polysaccharide form, its hydrolysis into fermentable sugars demanded the use of cellulolytic microorganisms (Seo et al., 2013). Cellulolytic microorganisms including bacteria and fungi have the capability to produce cellulases which are responsible for effective degradation of cellulose into its monomeric form viz. glucose (Immanuel et al., 2006). Cellulases comprising of endoglucanase (endo-1,4-β-D-glucanase), cellobiohydrolase (exo-1,4-β-D-glucanase) and β-glucosidase (1,4-β-D-glucosidase) has been of increasing curiosity due to their various applications in processing of fruit juice extraction, paper, textile industries, conversion of renewable cellulosic biomass, pharmaceuticals as well as improving the nutritive quality of bakery products and production of animal feed additives (Bai et al., 2012; Islam and Roy, 2018). These specific enzymes are also involved in production of bioethanol, lactic acid and single-cell protein from lignocellulosic agricultural residues. Moreover, endoglucanases are generally responsible for hydrolyzing the internal glycosidic bond of cellulose (Zafar et al., 2014).

There has been broad survey of fungal species which are primarily used for commercial production of cellulolytic enzymes (Maki et al., 2009; Sirohi et al., 2013). However, bacterial cellulase possesses more advantage due to higher growth rate of bacteria, expression of multienzyme complexes, stability under extreme conditions of temperature and pH and ability to withstand variety of environmental stresses (Deka et al., 2013). As a result, exploration of a group of microorganisms such as genus *Bacillus* paved the way to get into intense research which has capability of producing a large quantity of extracellular cellulolytic enzymes with increased specific activities and higher catalytic efficiency. The genus *Bacillus* is characterized by Gram-positive, aerobic or facultative anaerobic, rod-shaped, spore formers that have ability of producing cellulases (Ghani et al., 2013; Elshaghabee et al., 2017). These spore-forming bacteria represent a major microflora in many natural biotopes, where they play an important role in ecosystem development and transform many chemical compounds. Furthermore, cellulose degrading *Bacillus* sp. proved to be a potential probiotic candidate which can perk up aquaculture water quality and feed digestibility of aquaculture animals (Dat et al., 2019). Keeping in view towards agro-industrial applications of the genus related to cellulases, the study was undertaken to isolate, characterize and identify the cellulase positive *Bacillus* spp. from pond sediment samples.

MATERIALS AND METHODS

Sampling and isolation of *Bacillus* species

Twenty pond sediment samples were collected from fish culture ecosystems in and around the ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar. One gram of each collected samples was transferred to test tubes containing 10 ml of sterile normal saline solution. The tubes were boiled at 80℃ for 15 min in a rotary shaker bath at 50 rpm/min. Then from different samples 1ml was transferred to tubes containing 9 ml sterile NSS for ten-fold serial dilution. From each dilution of the respective samples 50 µl was taken and spreading was performed over nutrient agar plates. The plates were incubated at 37℃ for 24 hrs. After incubation different colonies were observed and morphologically distinct colonies were separated. To confirm the isolated colonies as *Bacillus* spp. Gram staining and endospore staining was carried out as per the standard microbiological procedures.

Qualitative cellulase activity assay

The positive isolates were further characterized by plate assay for CMCase to detect the extracellular enzyme cellulase responsible for the degradation of the complex polysaccharide cellulose as per the earlier methods (Teather and Wood., 1982). Tryptone soya broth was prepared in test tubes and all the positive isolates were inoculated in test tubes and incubated at 37℃ overnight. The next day the broth was centrifuged at 5,000 rpm for 20 min and the supernatant was collected. The medium having N/5 nutrient broth (HIMEDIA), 1% Carboxymethyl cellulose sodium salt (HIMEDIA) and 2% Agar powder (HIMEDIA) was prepared and wells of equal diameter were made over it. Exactly 100µl of the respective overnight grown supernatant in TSB was added in the wells and incubated at 37℃ for 48 hrs. After incubation the plates were treated first with 1% congored for 15 min, excess stain was discarded and then 1N NaCl was added for 15 min to see the zone of hydrolysis around the well. A clear zone surrounding the culture supernatant with reddish background indicates a positive reaction. The zone diameter was measured and plate photograph was documented.

Maintenance of pure cultures

The culture was maintained on CMC agar plates in dilution streaking and subculturing. For long-term storage the positive pure cultures were preserved in aliquots of 20% sterilized glycerol in eppendroff tubes. These were properly sealed with parafilm and stored at -20 ℃ for further use.

Morphological and biochemical characterization of the isolates

a. Morphological characteristics

The positive isolates were morphologically characterized following the standard microbiological methods from the bacteria grown on nutrient agar media. The size, form, colour, margin, elevation, consistency, pigmentation and opacity were recorded from isolated colonies. Staining characteristics of the isolates was performed by Gram staining and endospore staining. The staining characteristics, cell arrangement, endospore position etc. were recorded

b. Biochemical characteristics

The isolated bacteria were subjected to biochemical tests for tentative identification up to species level. Biochemical characters of the organisms were checked following the standard methods for identification of the isolates (Collee and Miles, 1989; Lacey, 1997). The isolates were characterized for catalase, oxidase, indole production, methyl red, Voges Proskauer, citrate, nitrate, urea, motility, phenyl deaminase test as per the standard microbiological identification procedures.

Carbohydrate utilization test

Utilization of carbohydrates and related compounds such as arabinose, glucose, sucrose, raffinose, trehalose, salicin, etc. and resultant acid production was assessed by the process. The test was performed on phenol red broth base medium with 1% agar along with using 1% filter sterilized respective sugars and sugar alcohols by the isolates. Yellow coloration of the medium was considered positive test while negative test was indicated by no colour change of the medium (Hassan, 2018). The isolates were incubated at 37℃ and each day the colour was observed. Tubes showing colour change were recorded and discarded where other tubes were kept for five days to confirm false negative results

Extracellular enzyme activity

The ability of the microorganisms to hydrolyze starch, casein, lecithin, gelatin and tributyrin for the presence of extracellular enzymes amylase, caseinase, lecithinase, gelatinase and lipase respectively were tested using N/5 nutrient broth supplemented with 1% of each substrate. These tests were performed by spot inoculating 24 hrs young cultures on plates containing respective substrates. After incubation the plates were treated with iodine vapour for amylase activity. For gelatinase activity the plates were treated with 1N H_2SO_4 saturated with sodium sulphate. Whereas for lecithinase activity the plates were checked for clear opalescence zone around the colony. In all other tests a clear zone surrounding the microbial colonies indicates a positive reaction. In the negative reaction no zone appears with the treatment.

Physiological growth Parameters

a. Growth at different concentration of NaCl

The cultures were streaked on nutrient agar plates with sodium chloride (NaCl) of varying concentration, i.e., 2.0%, 5.0%, 7.0% and 10%. All the plates along with a control nutrient agar plate without NaCl were incubated at 37℃ for 18-24 hrs. The maximum concentration of NaCl up to which growth occurred was considered as the maximum salt tolerance of the isolates.

b. Test for growth at different temperatures

The culture was revived in nutrient broth. 100µl of this overnight culture were dispensed into different tubes containing 10ml of nutrient broth. The growth of the isolates was tested on tubes by incubating them at different temperatures viz. 30℃, 37℃, 42℃, 45℃, 50℃, 55℃, 60℃, 65℃. The minimum and maximum temperature at which growth occurred were taken as the lower and upper temperature limits of the organisms, respectively.

Identification of the isolates

Comprehensive results of morphological, biochemical and physiological characteristics of the isolates were used for identification of the isolates matched with the Bergey's manual of Systematic Bacteriology (Vol-II).

RESULTS AND DISCUSSION

Isolation of cellulase positive *Bacillus* spp.

Out of 20 different sediment samples collected, a total of 68 different isolated colonies were randomly picked up from nutrient agar plates and further processed for Gram reaction and endospore staining. Out of the isolates, 16 isolates showed rod shaped microscopic character and Gram-positive reaction, for which endospore staining was carried out. Endospore staining showed all the isolates to be positive for presence of endospore and in some species also swollen sporangia were observed. Further these isolates were tested for endoglucanase activity by a qualitative approach using congored polysaccharide interaction assay (Teather and Wood., 1982). Surprisingly, all the isolates were found to be positive for the CMCase activity. The isolates were designated as RJ1 to RJ16. The colony characteristics, gram reaction and CMCase activity of a positive isolate is presented in Fig.1 [A], [B], [C].

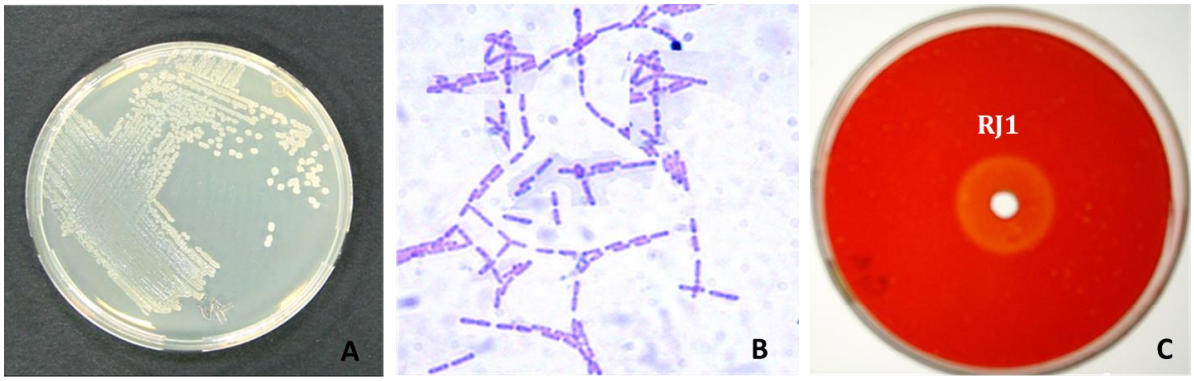

Fig. 1. Photograph of a *Bacillus* sp. Isolate (RJ1) showing colony characteristics [A], Gram reaction [B] and CMCase activity [C].

Biochemical, enzymatic and carbohydrate fermentation characterization of the isolates

In biochemical characterization all the isolates showed positive for catalase and oxidase activity, owing their identity as aerobic microorganisms. All the isolates were tested negative for indole and H_2S production. In tests like citrate utilization, methyl red production and Voges Proskauer reaction variation in positive reactions are observed. Out of all the 16 isolates 7 isolates showed positive reaction for methyl red, 11 for Voges Proskauer test, 12 for citrate utilization. Similar observations were also observed in case of motility and nitrate reduction tests. The detailed characteristics of the isolates is presented in Table 1. and reaction features in Fig. 2 [A].

Table. 1.

Biochemical characteristics of the isolates

Isolates	Biochemical tests										Extracellular Enzymes				
	Catalase	Oxidase	Indole	MR	VP	Citrate	H_2S	Urease	Nitrate	Motility	Amylase	Caseinase	Gelatinase	Lecithinase	Lipase
RJ1	+	+	-	-	-	+	-	+	+	+	+	+	+	-	-
RJ2	+	+	-	+	-	-	-	+	-	-	-	+	+	+	-
RJ3	+	+	-	-	-	+	-	+	+	-	+	+	+	-	-
RJ4	+	+	-	+	-	-	-	+	-	-	-	+	+	+	-
RJ5	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-
RJ6	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-
RJ7	+	+	-	-	+	+	-	+	-	+	+	+	+	-	-
RJ8	+	+	-	+	+	-	-	+	+	-	+	+	+	+	-
RJ9	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-
RJ10	+	+	-	+	+	-	-	+	+	+	-	+	+	+	-
RJ11	+	+	-	+	+	-	-	+	+	-	-	+	+	+	-
RJ12	+	+	-	+	-	+	-	+	+	-	-	+	+	+	-
RJ13	+	+	-	+	+	+	-	+	-	+	-	+	+	-	-
RJ14	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-
RJ15	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-
RJ16	+	+	-	+	+	+	-	+	-	+	-	+	+	-	-

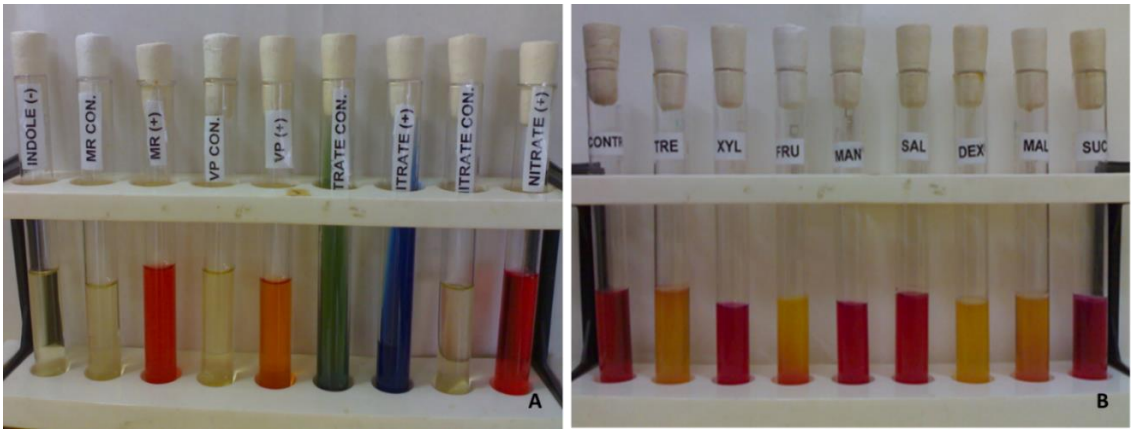

Fig. 2. [A] Biochemical characteristics of the isolates [B] Sugar utilization profile; red colour (Negative), yellow colour (positive)

For detection of extracellular enzymatic activities of the isolates, starch hydrolysis, casein hydrolysis, gelatin liquefaction, lecithinase test and lipase test were conducted. All the isolates were found positive for caseinase and gelatinase, whereas and 9 were amylase producers. 6 were lecithinase positive and all were lipase negative (Table 1). Sugar utilization tests revealed that metabolic fingerprint was very homogenous i.e. all of the isolates were glucose, fructose, trehalose and cellobiose utilizing strains. Maximum strains were mannose and arabinose positive with production of acid in the media whereas all most all strains were negative for melezitose, rhamnose and melibiose. The detailed carbohydrate fermentation pattern of the isolates is illustrated in the Table 2 and change in medium color in Fig. 2 [B].

Table. 2.

Different carbohydrate utilization pattern of the cellulase positive isolates

Isolates	*Mannose*	*Lactose*	*Fructose*	*Inositol*	*Trehalose*	*Maltose*	*Dextrose*	*Xylose*	*Arabinose*	*Mannose*	*Sorbose*	*Rhamnose*	*Cellobiose*	*Raffinose*	*Inulin*	*Sorbitol*	*Melibiose*	*Salicin*	*Galactose*	*Melezitose*	*Sucrose*
RJ1	-	-	+	-	+	+	+	-	+	+	-	-	+	-	-	+	-	+	-	-	+
RJ2	+	-	+	-	+	+	+	-	-	+	-	+	+	+	-	-	-	+	-	-	+
RJ3	+	-	+	-	+	+	+	+	+	+	-	-	+	-	-	+	-	+	-	-	+
RJ4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+
RJ5	+	-	+	+	+	-	+	-	+	+	-	-	+	-	+	-	-	-	-	-	+
RJ6	+	-	+	+	+	+	+	-	+	+	-	-	+	-	-	-	-	+	-	-	+
R	+	-	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-	-	-	-	+

RJ7																					
RJ8	-	-	+	-	+	-	+	-	-	-	-	-	+	+	-	-	-	+	-	-	+
RJ9	+	-	+	-	+	+	+	-	+	+	-	-	+	+	-	-	-	-	-	-	+
RJ10	+	-	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	-	-	+
RJ11	-	-	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	+	-	+
RJ12	+	-	+	-	+	-	+	+	-	+	-	-	+	+	-	+	-	+	-	-	-
RJ13	-	-	+	-	+	-	+	-	+	+	-	-	+	-	-	-	-	+	+	-	+
RJ14	+	-	+	-	+	-	+	+	-	+	-	-	+	-	-	-	-	+	-	-	+
RJ15	+	-	+	-	+	-	+	+	-	+	-	-	+	-	-	-	-	+	-	-	+
RJ16	+	-	+	-	+	-	+	-	+	+	-	-	+	+	-	-	-	+	+	-	+

Physiological characteristics

The physiological response of the isolates was tested for salt and temperature tolerance. Salt tolerance assay was performed at various concentrations at 2%, 5%, 7% and 10% using NaCl. Maximum of the isolates were tolerant up to 7% of salt concentration and at 10% NaCl all the strains were not able to grow except RJ1 strain (Table 3). Different temperatures at 30℃, 37℃, 40℃, 45℃, 50℃, 55℃, 60℃, 65℃ all the isolates were tested for their tolerance and maximum (56%) of the isolates were tolerant up to 60℃ and at 65℃ all the isolates didn't show any growth symptoms. All the isolates were observed to grow up to 45℃ whereas beyond the range variation in growth parameters were observed (Table 3).

Table. 3.

Effect of NaCl concentration and temperature on growth characteristics of the *Bacillus* isolates

Isolates	Salt Concentration				Temperature							
	2%	5%	7%	10%	30℃	37℃	40℃	45℃	50℃	55℃	60℃	65℃
RJ1	+	+	+	+	+	+	+	+	+	+	+	-
RJ2	+	+	-	-	+	+	+	+	+	+	+	-
RJ3	+	+	+	-	+	+	+	+	+	+	+	-
RJ4	+	+	-	-	+	+	+	+	-	-	-	-
RJ5	+	+	+	-	+	+	+	+	+	+	+	-
RJ6	+	+	+	-	+	+	+	+	+	-	-	-
RJ7	+	+	+	-	+	+	+	+	+	-	-	-
RJ8	+	+	-	-	+	+	+	+	+	+	+	-
RJ9	+	+	+	-	+	+	+	+	+	+	+	-
RJ10	+	+	-	-	+	+	+	+	-	-	-	-
RJ11	+	+	-	-	+	+	+	+	-	-	-	-
RJ12	+	+	-	-	+	+	+	+	-	-	-	-
RJ13	+	+	+	-	+	+	+	+	-	-	-	-
RJ14	+	+	+	-	+	+	+	+	+	+	+	-
RJ15	+	+	+	-	+	+	+	+	+	+	+	-
RJ16	+	+	+	-	+	+	+	+	+	+	+	-

Species identification

Comprehensive results of biochemical, enzymatic and physiological characters of the isolates were matched with the Bergey's manual of Systematic Bacteriology (Vol-II) and identified as different species of *Bacillus* spp. Among the isolates 6 isolates were identified as different strains of *Bacillus cereus*, 2 isolates as *B. pumilis* whereas majority of rest isolates were identified to be *B. subtilis* (Table 4).

Table. 4.

Identified organisms on the basis of conventional biochemical tests

Isolates	Identified as	Isolates	Identified as
RJ1	*Bacillus subtilis*	RJ9	*Bacillus subtilis*
RJ2	*Bacillus cereus*	RJ10	*Bacillus cereus*
RJ3	*Bacillus subtilis*	RJ11	*Bacillus cereus*
RJ4	*Bacillus cereus*	RJ12	*Bacillus cereus*
RJ5	*Bacillus. subtilis*	RJ13	*Bacillus pumilis*
RJ6	*Bacillus subtilis*	RJ14	*Bacillus subtilis*
RJ7	*Bacillus subtilis*	RJ15	*Bacillus subtilis*
RJ8	*Bacillus cereus*	RJ16	*Bacillus pumilis*

After identification it was observed that all the isolates belong to *B. cereus* showed a positive reaction towards lecithinase activity whereas both *B. subtilis* and *B. pumilis* showed negative for lecithinase. In a similar study Mohanty et al., 2011, also reported the presence of lecithinase activity in *B. cereus* species. Our findings corroborated with the study of Tariq et al. (2016) where *B. subtilis* isolates were found to be capable of hydrolyzing starch, casein, gelatin, cellulose by producing zone of hydrolysis around the colonies. The authors of the present study reported that confirmed eight *B. subtilis* is indole and methyl red negative. Lu et al. (2018) supported the present result where they found same species to be indole and methyl red negative. Another observation was marked in temperature tolerance where majority of the *B. cereus* isolates were detected to be less heat tolerant as compared to both other species. Colony morphology in nutrient agar plates showed a smooth colonial characteristic for *B. cereus* isolates but for *B. subtilis* and *B. pumilis*, rough irregular colonial morphology was observed. Bai et al. (2013) reported that colonial morphology of *B. subtilis* displayed rough, opaque, fuzzy white or slightly yellow with jagged edges when grown in nutrient agar. In terms of zone size for cellulase activity in CMC agar plates *B. subtilis* isolates were found to be owing higher zone of hydrolysis in comparison to *B. pumilis* and *B. cereus*. Kim et al. (2012) investigated good colonial development with highest carboxymethyl cellulase activity of *B. subtilis* species isolated from agricultural environments. In another study, *Bacillus* strains (P3-1 and P4-6) produced maximum cellulase activity at 0.015 U ml^{-1} in comparison to *Paenibacillus* species isolated from soil sample (Akaracharanya et al., 2014). The study indicates that *Bacillus* spp. to be the dominant microflora in pond sediment samples as far as cellulase activity is concerned. A clearer identification strategy employing 16S rDNA PCR sequencing approach for rapid and accurate species identification.

CONFLICT OF INTERESTS

We declare that no competing interests exist among the authors of this article.

ACKNOWLEDGEMENTS

Authors would like to acknowledge ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Odisha for providing all laboratory facilities and Odisha University of Agriculture and Technology, Bhubaneswar, Odisha for necessary support to carry out the research work smoothly.

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