

An Insight into the Cellulolytic Potential of Three Strains of *Bacillus* Spp. Isolated from Benthic Soil of Aquaculture Farms in East Kolkata Wetlands, India

Arijit De¹, Souryadeep Mukherjee²,
Gobinda Chandra Sadhukhan³ and Nimai Chandra Saha^{4*}

¹Department of Zoology, Vidyasagar College, Block CL, Plot 3-8 & 44-50, Sector II, Salt Lake, Kolkata, West Bengal- 700 091, India.

²Department of Life Sciences, Presidency University, 86/1, College Street, Kolkata, West Bengal - 700073, India.

³UGC – HRDC, Jadavpur University, Block - LB, Plot No 8, Sector III, Salt Lake City, Kolkata, West Bengal 700098 Kolkata - 700098, India.

⁴University of Burdwan, Rajbati, Burdwan, West Bengal - 713104, India.

<http://dx.doi.org/10.22207/JPAM.12.3.66>

(Received: 10 June 2018; accepted: 26 August 2018)

The present study investigates the cellulolytic potential of three prokaryotic strains belonging to genus *Bacillus* isolated from the benthic soil of two aquaculture farms in East Kolkata Wetlands. For the purpose of identification and phylogenetic analyses, biochemical characterization of these three strains were performed followed by molecular characterization of 16S rRNA sequences. Maximum cellulolytic potential was found in *Bacillus cereus* Strain SWA6a for both Exo - β ,1-4- glucanase and Endo - β ,1-4- glucanase with an activity of 0.08214 ± 0.00412 and 0.11263 ± 0.00478 IU ml⁻¹min⁻¹ respectively. The enzyme activity ranged from 0.06112 to 0.08214 IU ml⁻¹min⁻¹ for Exo - β ,1-4- glucanase and 0.10018 to 0.11263 IU ml⁻¹min⁻¹ for Endo - β ,1-4- glucanase. Degradation of free insoluble cellulose ranged from 55 to 63% within a time span of 96 hours in pH 6.8 – 7.2 when incubated at 37°C. Overall, the cellulolytic activity of the isolated strains may have strong implications in the field of biological conversion of cellulose-containing municipal wastes, agricultural and forestry wastes. These isolates maintain the equilibrium of the aquaculture pond by maintaining the biogeochemical cycle of carbon and hence has the prospect to be used for bioaugmentation purposes as a step towards a microbial-based aquaculture method.

Keywords: Aquaculture benthos, *Bacillus*, Cellulolysis, 16S rDNA Analysis, Cellulase.

The process of photosynthesis converts light energy into chemical energy and thus results in the production of plant biomass with cellulose as a major component ¹. Thus as one of the most abundant materials on Earth, cellulose plays a key role in the material flow of this planet. Cellulose is one of the major renewable resources and is considerably available in agricultural and municipal wastes, however, most practiced disposal method of this waste is by burning which

not only eliminates the possibility of recycling but also pollutes the environment. ^{1,2}. Hence, research has been directed towards the study of the natural process of cellulose decomposition. The biogeochemical cycle of carbon completes with the degradation of the cellulose by microorganisms of the soil and guts of animals. Cellulase, an inducible enzyme is synthesized and secreted extracellularly by a diversity of microorganisms, including bacteria and fungi during their growth in cellulosic materials ³. Cellulose is decomposed by the microorganisms with a multi-enzyme system which converts it into its monomeric

* To whom all correspondence should be addressed.
E-mail: ncsvcbu@gmail.com

form of glucose. The enzyme system comprises Endo- β -1,4 glucanase which randomly cleaves β 1,4 linkage and extracts elementary fibrils from cellulose crystals, followed by Exo- β -1,4 glucanase which cleaves the non-reducing end of the cellulose fibrils and converts it into Cellobiose or Cellodextrose which is finally hydrolysed to glucose by β -Glucosidases⁴. In the present study, the focus is drawn towards the prokaryotic genera only. There are numerous references of cellulose degrading bacteria from soil sources such as manure compost⁵, tea garden soils⁶, the soil of paddy field⁷, etc. These studies report several genera of bacteria like *Bacillus*, *Acinetobacter*, *Clostridium*, *Paenibacillus*, *Trichonympha*, *Actinomycetes*, etc.^{7,8} with various degradation ability and enzymatic activity. Cellulose degrading bacteria have also been isolated from animal guts such as insect caterpillar and snail¹, termites⁹ and grass carp¹⁰.

This phenomenon of cellulose decomposition is important not only ecologically but also economically from the human perspective. Deeper insight into the cellulolytic microcosm can provide plausible remedies in the management of pollution caused by Solid Municipal Wastes¹¹. On the other hand, depletion of non-renewable sources of energy is a matter of utmost concern to humanity. To solve this problem, plant biomass is the only foreseeable source that has a sustainable future. Cellulosic materials in this context are enticing because of its low cost and plentiful supply. Apart from these, the cellulolytic microorganisms are also found convenient in their industrial application where cellulose is used as a raw material. The most important of the industrial application may be the prospective use of lignocellulosic biofuels, where cellulosic materials would be used as a raw material for the production of sugars that can be converted into ethanol and other liquid fuels. The whole process would incorporate the use of microorganisms especially the prokaryotes to facilitate this conversion¹². Other notable industrial application of the cellulose degrading bacteria are found in the composting, paper industry, breweries and food industries¹³.

However, data of the prokaryotes with cellulolytic potential from the benthic soil of aquatic ecosystems are scanty. The only related study has been done on mangrove soils of

Mahanadi river delta, India which reports genera of bacteria like *Micrococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Xanthomonas* spp. and *Brucella* spp. as potential cellulolytic strains¹⁴. The study of the prokaryotic microcosm of the benthic soil of aquatic ecosystems is of utmost importance as they play a wide array of roles in the maintenance of the ecosystem. Recent advancements in the development of the aquaculture systems promote the idea of microbial-based culture systems over traditional culture methods. The Microbial-based culture system involves the study of the benthic soil which can lead to a better understanding of the roles of the members of the prokaryotic community in the microcosm of the aquaculture system¹⁵. Banking on this idea, this study investigates the benthic soil of two aquaculture farms in East Kolkata Wetlands in search of prokaryotic strains with cellulolytic potentials which can be used in the designing of a sustainable aquaculture system.

(Data in this paper are from first author's thesis to be submitted shortly for the partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Department of Zoology, The University of Burdwan, West Bengal, India.)

MATERIAL AND METHODS

Culture Media

All reagents used in the study were of analytical grades procured from HIMEDIA (India) and Sigma Aldrich Chemical (USA). Minimal salt media (MSM) was used for culture of bacterial strains as a source of essential inorganic nutrients. Composition of the MSM (per litre) was $(\text{NH}_4)_2\text{SO}_4$, 2g; Na_2HPO_4 , 3.61 g; KH_2PO_4 , 1.75g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 50mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 μg ; H_3BO_3 , 10 μg ; $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$, 10 μg ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 70 μg ; MoO_3 , 10 μg ; pH adjusted to 7.0.

Regular Culture and subculture were done with nutrient agar medium having Peptone 5 g, NaCl 5 g, Beef extract 1.5 g, Yeast extract 1.5 g, Agar 2%, pH 7.4 \pm 0.2 (per litre) and Luria agar having Casein enzymic hydrolysate 10g, Yeast extract 5g, Sodium chloride 5g, Agar 2%, pH 7.0 \pm 0.2 (Per litre) used after autoclaving (120°C temperature and 20 psi pressure, 15 minutes).

Collection

Soil (~5g) was collected from two Aquaculture farms in East Kolkata Wetlands (22.5528° N, 88.4501° E). Soil samples were collected from the top layer of the benthic soil under 60 cm of water. Soils were aseptically transferred to sterile containers. The containers were labelled and taken to laboratory for further processing.

Isolation

Bacterial isolation was done following the protocol of Gupta et al., 2012,¹ with minor modifications. 2g of soil was inoculated in 50 ml Minimal salt media (MSM) with 2 % w/v Carboxymethylcellulose (CMC) as the sole carbon source. The culture was maintained for 7 days at 37°C with constant shaking at 120 rpm.

The culture media was then subjected to serial dilution up to 10⁶ times and was spread on Congo Red agar media KH₂PO₄ 0.5 g, MgSO₄ 0.25 g, CMC 20 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1 L and at pH 6.8–7.2. This media is a fast process to test the cellulolytic activity via discoloration of Congo red around the colonies as halos. After incubation for 12 hours at 37°C, single colonies of the isolates were streaked in Congo red agar media. The colony size and diameter of the halo are noted. The Hydrolysing Capacity (HC) of the isolates were calculated using the ratio of the diameter of the halo and diameter of the colony. This method was used to determine the best isolates with considerable cellulolytic potential and were selected for further studies. In this study three strains outperformed other isolates and were selected for further enzymatic analyses.

The three selected isolates were cultured in an enzyme production media composed of MSM supplemented with sterilised Filter paper (@ 0.05g / 20 ml, cut into 1cm² pieces) for Exo-β-1,4 glucanase assay and the same media containing CMC (0.5 g / 20 ml), at pH 6.8–7.2 for Endo-β-1,4 glucanase assay, and the cultures were incubated for 96 hours at 37°C in a shaker incubator at 120 rpm.

After the incubation period, the cultures were subjected to centrifugation at 8000 rpm for 10 mins at 4°C. The supernatant was collected, filter sterilised and was stored at 4°C for future enzymatic assays. The pellet of the media supplemented with Filter paper was collected to

analyze the degradation percentage by Gravimetric Analysis¹.

Enzyme Assay

The end product of the cellulose digestion is reducing sugar⁴, thus, total cellulase activity was estimated by measuring the amount of reducing sugar formed from CMC and filter paper. The enzyme activity was determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) Commission on biotechnology following the protocol given by Rajoka and Malik, 1994¹⁶ with minor modifications. The Exo-β-1,4 glucanase activity of the bacterial isolates were determined by incubating 0.5ml of the sterilised supernatant collected as mentioned earlier, with 1ml of 0.1M citrate buffer pH-4.8 and sterilised Whatman no1 filter paper (50mg) in the form of 1cm² pieces for 1hour at 50°C. The reaction was stopped by adding 3 ml of the 3,5-dinitrosalicylic acid (DNS) reagent to the reaction mixture. DNS added reaction mixture was boiled for 15 minutes and 40% sodium potassium tartrate was added to it. After cooling to room temperature, absorbance was measured at 550 nm (Systronics R2202 Double Beam Spectrophotometer) against blank with same constituents as above except the supernatant which was replaced with sterile triple distilled water of same volume.

Endo-β-1,4 glucanase activity was assayed by measuring the amount of reducing sugar from Carboxymethylcellulose and was determined by a similar process as that of Exo-β-1,4 glucanase, except the absorbance, was measured at 540 nm against blank¹⁶. The OD value thus obtained was then put into the equation as given below to obtain the activity of the enzyme in IU ml⁻¹min⁻¹.

Enzyme Activity (IU ml⁻¹min⁻¹) = Absorbance x Standard factor

A standard estimation was done using known concentration gradient of glucose (0.1 – 1.0 mg ml⁻¹, 10 samples at 0.1 mg ml⁻¹ interval) and following the same principle as stated above. The OD value thus obtained was used to calculate the standard factor. The same procedure was followed once for Endo-β-1,4 glucanase at 540nm and for Exo-β-1,4 glucanase at 550 nm. The Standard factor was calculated according to the following formula and finally, the mean value of the Standard factor was taken for enzyme activity estimation.

Standard Factor = Concentration of Glucose standard (mg ml⁻¹) / Absorbance (at 540 or 550nm)

pH and Temperature stability

Cell-free supernatants containing the crude enzymes were tested for their optimal pH and thermostability. The same process of enzymatic analysis was done by varying the pH of the enzyme production media and temperature of the enzyme prior to incubation. The pH was tested within a range of 4–10 (7 samples with an interval of 1 pH) and temperature were tested from 20°C to 70°C (6 samples with 10°C interval).

Biochemical Characterisation

Biochemical characterisation of the bacterial isolates were done by an array of tests including MR, VP, Motility, Gram's Staining, Citrate, Amylase, Oxidase, Catalase etc. were done to characterize the strains according to Bergey's Manual of Systematic Bacteriology¹⁷.

Molecular Characterisation

Genomic DNA isolation and molecular characterization of the strains

For the purpose of molecular characterization, genomic DNA was isolated from

Table 1. Biochemical Tests Result of the Isolates. The Positive result is represented with + symbol and negative with – symbol. Y and R represents Yellow and Red colour of the indicator in TSI media. Yellow indicates that the strains are able to ferment sugars in aerobic (Slant) or anaerobic (Butt) condition whereas Red indicates negative result. W stands for white colony in MacKonkey media which indicates these strains were able to utilise lactose

Biochemical Tests		SWO4	Strains SWO13a	SWA6a
TSI	Slant	Y	Y	R
	Butt	R	Y	Y
	H ₂ S	-	-	-
	Gas	-	-	-
Oxidase		+	+	+
Catalase		+	-	-
Gram Staining		-	-	+
KOH String Test		-	-	+
MR		+	-	-
VP		-	-	+
Gelatinase		-	-	+
Indole		-	-	-
Motility		-	-	+
DNase		-	-	-
MacKonkey		W	W	-
Urease		-	+	+
Citrate		+	-	-
Amylase		+	+	-

Table 2. The Closest Relative, Max Score and Identity % obtained from the BLASTn analysis of the three isolates. The GenBank accession numbers of the strains are also mentioned

Isolate	Closest Relative	Max Score	Identity (%)	Accession Number (GenBank)
SWO4	<i>Bacillus flexus</i>	1753	98%	MG461558
SWO13a	<i>Bacillus cereus</i>	1430	97%	MF554692
SWA6a	<i>Bacillus cereus</i>	1555	99%	MF554643

the strains by Bacterial Genomic DNA isolation kit (GCC Biotech, India). PCR amplification of the 16S rRNA gene DNA was carried out in a thermal cycler (Biorad Gradient Thermal Cycler) using 200 µl PCR tubes with a reaction mixture volume of 50 µl. Each of the reaction mixtures contained 2 µl of template DNA, 1 µl of forward primer 27f (5'-AGAGTTTGATCMTGGCTCAG-3'), 1 µl of reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3'), 25 µl of 2X PCR MasterMix (Thermo Fisher Scientific), and nuclease-free sterile water to 50 µl. The reaction mixture was subjected to an amplification of 30 cycles, each of which consisted of three steps in the following order, denaturation of template DNA at 94°C for 1 min, annealing of the template DNA at 50°C for 1 min and extension of the primers at 72°C for 2 min. The PCR products were then subjected to Agarose Gel Electrophoresis at 1.5% gel using Ethidium Bromide stain and were subsequently visualized under UV light. Amplicons were purified by Agarose Gel purification kit (New England Biolabs) and sequenced by using the paid sequencing facility from Xcelaris (Ahmedabad, India).

Identification and Phylogenetic analyses

The Sequences obtained were analyzed using BLASTn in the NCBI database (<http://>

blast.ncbi.nlm.nih.gov/Blast.cgi). The results thus obtained were screened and ten nearest neighbors were chosen based on the Highest Max Score and Total Score. The aligned sequence files were downloaded and further alignment and phylogenetic analyses were done in MEGA7¹⁸. The alignment of the sequences with their nearest neighbors was done using CLUSTALW. To eliminate the possibility of incorporation of potential chimeras, the sequences were checked using online webtool DECIPHER¹⁹. The phylogenetic tree was constructed based on the evolutionary distances calculated from the number of base substitutions per site of all the three strains by the Neighbor-joining method²⁰ using Maximum Likelihood Composite model²¹. The difference in composition bias was considered in evolutionary comparisons during the construction of the Phylogenetic tree²².

Statistical analysis

All experiments were performed at least in triplicate and values are expressed as mean values \pm standard deviation (S.D.)]. Statistical analyses were done and means were compared by Student's t-test using SPSS 17.0 (SPSS Inc., Chicago, USA). Differences between means at ($P \leq 0.05$) level were considered significant.

Table 3. The results of screening tests in Congo Red Agar. The Diameter of the Clearing Zone and that of the Colony are shown. The HC gives an estimation of the cellulolytic potential of the three strains

Strain	Diameter of the Clearing Zone (Halo) (in mm)	Diameter of the Colony (in mm)	Hydrolysing Capacity (HC)
SWO4	12.1	20.8	1.71
SWO13a	8.7	17.9	2.05
SWA6a	9.3	24.2	2.6

Table 4. The Enzymatic activities and Cellulose Degrading ability of the isolates. The Mean values for each strain is given with SD mentioned. The Degradation of the Filter paper indicates the Cellulolytic potential of the strains

Strains	Activity (IU ml ⁻¹ min ⁻¹)		Degradation of Filter Paper (%)
	Exo - β ,1-4- glucanase	Endo- β ,1-4- glucanase	
SWO4	0.06112 \pm 0.00326	0.10018 \pm 0.00269	55.1 \pm 2.34
SWO13a	0.07924 \pm 0.00510	0.10301 \pm 0.00156	61.9 \pm 1.25
SWA6a	0.08214 \pm 0.00412	0.11263 \pm 0.00478	63.4 \pm 0.98

RESULTS

Characterisation of Cellulose degrading Strains

The three isolates were named as strains SWA4, SWA13a, and SWO6. Results of morphological characterization of these strains like colony shape, cellular morphology, gram character and detailed biochemical characteristics are provided in Table 1. The Results showed that SWO4 and SWO13a as a Gram-negative, non-motile strain whereas SWA6a is a gram-positive, motile strain. All the three strains were capable of producing enzymes like gelatinase, amylase, urease, citratase, catalase and oxidase other than

cellulase. The strains were capable of fermenting sugars with varying potentiality.

Molecular Characterisation

BLASTn analysis results are depicted in Table 2. Isolates SWO13a and SWA6a were identified to be as *Bacillus cereus* with 97% and 99% homology respectively. Isolate SWO4 was *Bacillus flexus* with 98% sequence homology. The Phylogenetic tree constructed is depicted in Figure 3. The analysis of the tree shows that the isolates are in separate lineages and are different from one another with SWO4 in a separate lineage altogether.

Screening test results

The Screening test result performed in Congo Red agar media revealed a glimpse of the

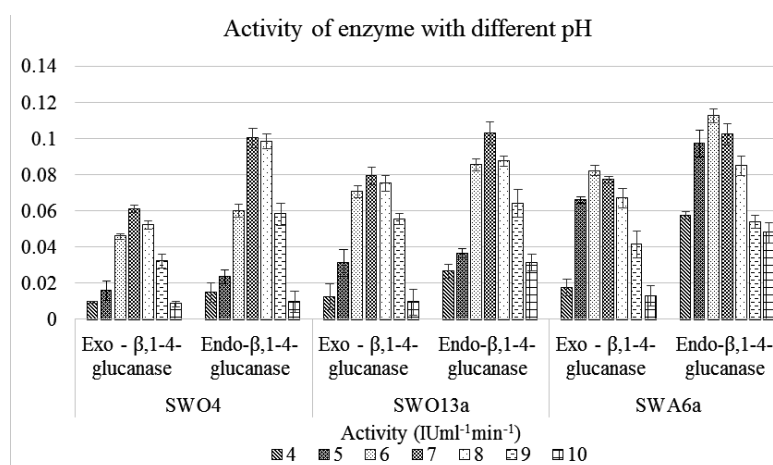


Fig. 1. Graphical representation of the variation of activity of Endo-β-1,4 glucanase and Exo-β-1,4 glucanase of the three Strains with varying pH ranging from pH 4 -10

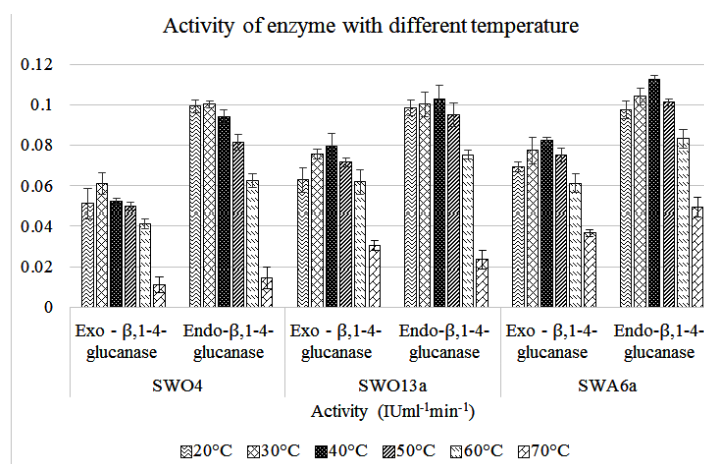


Fig. 2. Graphical representation of the variation of activity of Endo-β-1,4 glucanase and Exo-β-1,4 glucanase of the three Strains with varying Temperature ranging from 20°C - 70°C

comparative account of cellulolytic potential of the three strains involved and is given in Table 3. The highest HC value was found in Isolate SWA6a and the lowest was given by SWO4. However, it is mention worthy that these three strains excelled other strains that were subjected to the same test

and hence was selected for the enzymatic assay.

Enzyme Assay

The enzyme assay confirms the result obtained in the screening test and is given in Table 4. Isolate SWA6a scores the best enzymatic activity for both Exo - β ,1-4- glucanase and Endo - β ,1-4-

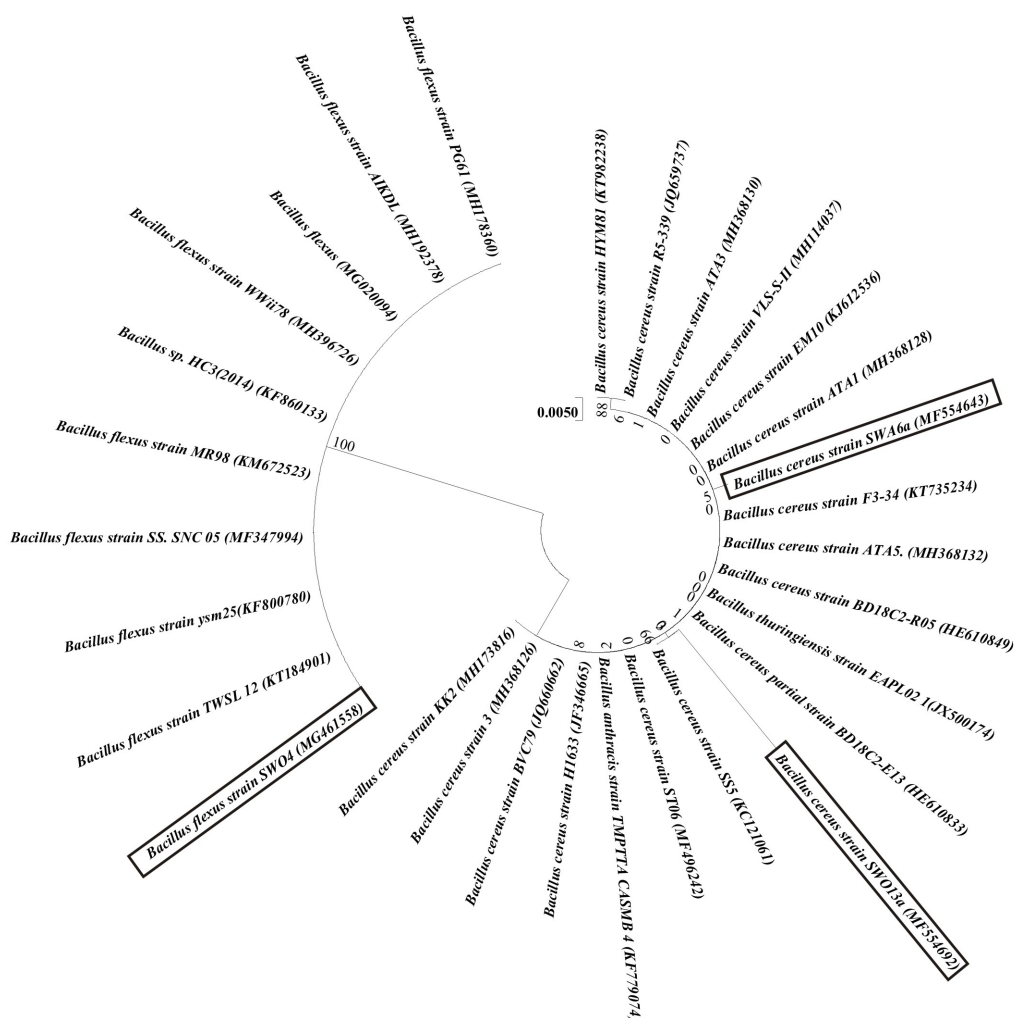


Fig. 3. Evolutionary relationships of the three strains. The Genbank Accession Numbers for each strains used in this analysis is mentioned in parenthesis. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.10455038 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 30 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 829 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

glucanase with an activity of 0.08214 ± 0.00412 and 0.11263 ± 0.00478 IU ml⁻¹min⁻¹ respectively. The Overall enzyme activity ranged from 0.06112 to 0.08214 IU ml⁻¹min⁻¹ for Exo - β ,1-4- glucanase and 0.10018 to 0.11263 IU ml⁻¹min⁻¹ for Endo - β ,1-4- glucanase. Gravimetric analysis revealed that isolates SWO4, SWO13a, and SWA6a were able to degrade 55.1%, 61.9% and 63.4% of raw cellulose given in the form of filter paper respectively, in a span of 96 hours under the given conditions.

Results of pH and Temperature stability estimation of both the enzymes of the three isolates are depicted graphically in Figure 1 and 2 respectively. Both the analyses showed a common pattern for both the enzymes. For pH, the maximum activity of the enzymes obtained from the strains showed the highest activity within a range of 6–8 and diminishing at either extremities. In case of temperature the highest activity was recorded within 30 – 40°C with the same trend of diminishing activity with either increase or decrease of the temperature. The isolate SWA6a not only showed the highest activity under optimum pH and temperature conditions but also was more temperature and pH tolerant when compared to the other two isolates.

DISCUSSION

Cellulose is indeed considered as a viable option for the supply of raw materials for several industries. Several research indicates its potentiality in this regard. However, the most environment friendly way to decompose and utilise this complex biomolecule is to degrade it using microorganisms like bacteria as a tool ²³. The past references showed varied degree of cellulolytic potential from unidentified strains with ranges of 0.012 to 0.196 IU ml⁻¹min⁻¹ for Exo - β ,1-4- glucanase and 0.1622 to 0.400 IU ml⁻¹min⁻¹ for Endo - β ,1-4- glucanase was estimated from bacteria inhabiting the gut of cellulose – feeding organisms ¹. Another study demonstrated the cellulolytic potential of *Bacillus licheniformis* isolated from Indian Hot springs to be 0.542 IU ml⁻¹min⁻¹ for Exo - β ,1-4- glucanase and 0.120 IU ml⁻¹min⁻¹ for Endo - β ,1-4- glucanase ²⁴. These two results correspond to the data obtained in the study. On the other hand, present findings do not correspond to the observations of Behera et al. who described a very high cellulolytic potential

(2.471 to 98.253 IU ml⁻¹min⁻¹ for Endo - β ,1-4- glucanase) produced by several genera of bacteria like *Micrococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Xanthomonas* spp. and *Brucella* spp. isolated from the benthic soil of Mahanadi river ¹⁴.

The results from various experimental protocols explored revealed that all the three isolates were capable of utilizing cellulose as a sole carbon source and their efficacy in the breakdown of cellulose is also mention worthy. Similar results were also obtained in the estimation of cellulolytic potential by the earlier workers ^{1,24}. The data seems to be the first report of prokaryotes with cellulolytic potential from the benthic soil of Aquaculture farms of East Kolkata Wetlands. It can be said that these bacteria are efficient in the decomposition of cellulosic materials in the aquatic ecosystem and hence maintains the ecological balance of the aquaculture ponds by maintaining a proper biogeochemical cycle of carbon providing an ecologically healthy environment for fish culture. This study also illuminates the prospective use of these strains in the cellulose dependent industry and that these strains have the potential efficacy. They can also be used in Solid Waste Management via composting method ¹¹. From the perspective of aquaculture, identification of such strains with cellulolytic potential paves the way for future implementation for microbial – based culture methods. This strains can also be bioaugmented in the natural aquaculture ponds with high level of plant debris and will ultimately lead to the sustenance of the pond.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Department of Zoology, The University of Burdwan and Department of Life Sciences, Presidency University for providing infrastructural facilities to carry out the work.

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