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Isolation, Characterization and Molecular Identification of Endophytic Bacteria from Mangrove Plants

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Abstract

Mangroves are plants that grow along coastal saline or brackish water, they occur worldwide in the tropics and subtropics, mainly between latitudes 25˚S. Microbes that live symbiotically within the plants are called endophytes. The aim of this study is to confirm the ability of endophytic bacterias in production of extracellular enzymes of economic importance. In the present study, 44 strains of endophytic bacteria were isolated from mangrove plants viz. *Rhizophora annamalaiana, Rhizophora appiculata, Suaedo monoica, Ceriops decandra, Rhizophora mucarneta*. The isolated bacterial strains were tested for various extracellular enzyme of industrial importance viz. Amylase, Gelatinase, Cellulase, Protease, Catalase, Pectinase, Chitinase, and Laccase. The order of enzymes activities found in this study for the isolated microorganisms is Amylase > Cellulase > Protease > Pectinase>Catalase > Gelatinase> Chitinase and Laccase. Growth medium was standardised to enhance enzyme activity. Effect of pH, and temperature on enzyme activities were tested.

Keywords

Mangrove leaves, endophytic bacteria, enzymes

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INTRODUCTION:

Mangrove is a unique forest, representing intermediate vegetation between land and sea. These plants grow in oxygen deficient, waterlogged soil. To survive in such harsh conditions mangrove have evolved a number of physiological and structural adaptations like Vivipary, Pneumatophores, Prop roots, etc. All mangrove species have mechanisms to provide air to their root system from the atmosphere. Hence, they can tolerate anaerobic conditions to some extent.

Endophytic microorganisms (fungi and bacteria) are those that inhabit plant tissues for a period of its vital cycle. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopathogens microorganisms (Azevedo et al., 1999) [1]. These microorganisms produce pharmacologically activate substances with biotechnological potential such as antitumor agents (pestalotiopsis microspora, taxol), antifungal agents (cryptosporiopsis criptocandina, quercine), besides producing factors of plant growth, toxins and enzymes. (Araujo et al., 2002; Strobel, 2002; Azevedo, 1998; Strierle et al., 1993) [2,3,4,5]. Microorganisms gains importance in enzyme production due to its high production capability, low cost and susceptibility to genetic manipulation. Actually, the enzymes of microbial origin have high biotechnological interest such as in the processing of foods, manufacturing of detergent, textiles, pharmaceutical products, medical therapy and in molecular biology (Pilnik and Rombounts, 1985; Falch, 1991; Rao et al., 1998) [6,7,8]. It is, therefore, necessary to find microorganisms that produce enzymes for specific substrate, with different requirements in temperature range, pH and presence of different ions, for different production processes (Falch, 1991) [7]. Endophytic microorganisms occupy a relatively unexplored site as a source in obtaining more enzymes with different potentialities.

The present study was carried out to isolate endophtyic bacteria from *Rhizophora annamalaiana, Rhizophora appiculata, Suaedo monoica, Ceriops decandra, Rhizophora mucarneta*.

MATERIALS AND METHODS:

Chemicals: All chemicals were procured from Himedia Ltd, Merck Ltd.

Collection of leaf

The leaves were collected from mangrove plants *Rhizophora annamaliana, Rhizophora appiculata, Rhizophora mucronata, Ceriops decandra* and *Suaeda monoica* of pichavaram mangrove forest, Tamilnadu, India.

ISOLATION OF ENDOPHYTIC BACTERIA

Endophytic bacteria were isolated as per the protocols of V.H.Sunitha et al 2013 [9]. The leaves were washed in running tap water, rinsed with sterile distilled water, followed by surface sterilisation using sodium hypocloride solution. The sterile leaves were cut into bits and placed on nutrient agar plate and incubated at 30°C for 24 hours. Pure cultures were isolated and maintained in nutrient agar. This was used for further studies.

MICROBIOLOGICAL CHARACTERIZATION

Biochemical tests.

The following tests were carried out to identify isolated bacteria: Gram straining test, Morphology test, Motility test, Spore test, Indole test, Methyl Red test, Voges-proskauer test, Citrate test, Catalase test, Oxidase test, Nitrate Reduction test, Urease test, Fermentation (Glucose) test, Fermentation (Lactose) test, Fermentation (Sucrose) test, H_2S Production test. The tests were carried out using standard protocols of Burbianka and Pliszka, 1983; Sneath, 1986; Slepecky and Hemphill, 2006 [10,11,12].

Molecular taxonomy of the potent isolates

Bacterium identification was conducted by the molecular method depending on gene phylogenetic approximation. PCR amplifications of the 16S rRNA gene were performed with 16S rRNA Eu-bacterial primer (50-GAGTTTGATCCTGGCTCAG-30; 50-AGAAAGGAGGTGATCCAGCC-30) following the method described by Relman (1993) [13]. The phylogenetic tree was constructed by utilizing the neighbour-joining method and assessed with 1000 bootstrap replications. The minimum value of the nucleotide similitude percentage to define the species at the taxonomic level was 98% (Rossello-Mora and Amann, 2001) [14]. The rRNA gene sequence obtained from the isolate Nrc-1 was compared with other bacterial sequences by using NCBI mega BLAST. The nucleotide sequence was aligned in CLUSTALX. The phylogenetic analyses were conducted using MEGA version 6 software (Tamura et al., 2007) [15].

SEM OBSERVATION:

Bacteria were also subjected for scanning electron microscope (SEM) (Ho et al 1999) [16]. The specimens were first fixed with 2.5% glutaraldehyde in phosphate buffer (pH6.8) was carried out followed by incubation for more than 4h in room temperature. The specimens were dehydrated by a graded series of ethanol (50,70,80,90,and 95%, 100) for about10 min at each step of ethanol for every 10 min and followed by 100% ethanol for 20 min and then freeze dried and coated with gold-palladium and were examined with a scanning electron microscope at different magnification under a SEM (Jeol,JSM-6390LV, Japan) at an accelerating voltage of 5kV.

GROWTH MEDIA DEVELOPMENT AND INOCULATION

Various parameters influencing the growth and production was evaluated by changing the proposition of the medium, and growth condition

Four different types of medium were used

1) 0.5% Peptone, 0.3% Yeast extract, 0.5% NaCl
2) 3.5% Peptone, 0.3% Yeast extract, 0.5% NaCl
3) 3% Glucose, 0.5% Peptone, 0.3% Yeast extract, 0.5% NaCl
4) 3.5% Peptone, 0.3% Yeast extract, 0.5% NaCl, 3%Glucose

ENZYMATIC ACTIVITY:

Qualitative method of enzyme activity

The productions of following enzymes were analysed: Amylase, Gelatinase, Keratinase, Chitinase, Pectinase, Cellulase, Catalase, Ligninase, Protease and Lipase.

Determination of Amylase activity:

Amylase activity was carried out using the methodology described by Hankin and Anagnostakis 1975, Aysha Jussara Ivonilde Carrim et al., 2006 [17] [18]. Briefly, culture was grown in nutrient agar plate

(NA) amended with 0.2% of starch, after incubation, the culture was treated with iodine which allowed the visualization of clear halo around the colonies.

Determination of Gelatinase Activity:

Gelatinase activity was carried out using the methodology described by Hankin and Anagnostakis(1975) [17]. Briefly, the isolate was inoculated in Nutrient Agar (NA) with 0.2% of Gelatin, after incubation, the culture showed clear halo around the colonies.

Determination of keratinase Activity:

The isolate was inoculated in sterile minimal medium with chicken feathers using the procedure of Vieria,1999 [19]. Feathers biodegradation indicated keratinase activity.

Determination of proteolytic Activity:

Proteolytic activity was determined using the protocols of Vieria,1999[19]. To determine the casein hydrolysis, skimmed milk agar (5g) was used, Microorganisms were inoculated and after the growth period the presence of clear halos around the colonies were observed.

Determination of Chitinase activity:

Chitin hydrolysis was determined using the protocols of Andro et al. (1984) [20], nutrient agar amended with chitin (0.5g) was used. Presence of halo zone around the colony indicated presence of chitinase.

Determination of cellulolytic Activity:

cellulase hydrolysis was determined using the protocols of Andro et al. (1984) [20], nutrient agar was amended with Sodium carbonyl methyl cellulose 0.5% and incubated for 24h. Congo red was flooded on the plates. Presence of colourless halo around the colony indicated presence of cellulase.

Determination of Pectinolytic Activity:

pectinase hydrolysis was determined using the protocols of Andro et al. (1984) [20], nutrient agar was incubated for 96hours. After that the plate was flooded with 5.0ml HCl (mol l-1). Presence of halo zone around the colony indicated presence of pectin.

Determination of Catalase Activity:

catalase hydrolysis was determined using the protocols of Andro et al. (1984) [20], 24h culture of bacterial colony was transferred to a glass slide, it was added with a drop of H_2O_2. Presence of bubbles indicated presence of catalases.

QUANTITATIVE METHOD OF ESTIMATION OF AMYLASE ACTIVITY BY STARCH METHOD

Amylase activity was quantified by method of Bernfeld (1955) [21] using 3,5-dinitrosalicylic acid.

In a test tube, the reaction mixture containing 1ml of soluble starch solution mixed with 1 ml of potassium phosphate buffer, pH 6.9 was mixed with 0.1ml of the cured enzyme source from one of the labelled conical flasks and incubation, 2ml of the DNS reagent was a0dded and the reaction terminated by immersing the tube in a boiling water (100°C) for 10 min, and was read at 540nm. One unit of enzyme that hydrolyses 1mg of starch/min under assay condition.

QUANTITATIVE METHOD OF CELLULASE ACTIVITY

Cellulase activity was quantified using protocols of Monga et al 2011[22]. In a test tube, the reaction mixture (1ml of soluble starch solution mixed with 1 ml of potassium phosphate buffer, pH 6.9) was mixed with 0.1ml of the crude enzyme, 2ml of the DNS reagent was added and the reaction was terminated by immersing the tube in a boiling water (100°C) for 10 min. This was read at 540nm. One unit of enzyme that hydrolyses 1mg of starch under assay condition Standard for supplied Maltose.

EFFECT OF pH

The effect of pH on amylase, cellulase, protease production was determined by culturing the bacterium in the media with different pH (1,3,5,7,9,11,13). After incubating for 24 hours, enzyme assay was carried out. (Shankar et al 2011) [23].

EFFECT OF TEMPERATURE

The effect of temperature on amylase, cellulase and protease production was carried out by incubating culture media at various temperature -20°, 40°,60°,80°and 100°C along with arbitrary control at 37°C. The enzyme assay was carried out after 24 hours of incubation (Sathees Kumar et al 2011) [24].

Quantitative method of Estimation of Protease Activity

ACID, ALKALINE AND NEUTRAL PROTEASE

The acid protease activity in crude enzyme extract was assayed according to the modified method (Srividya Shiva Kumar 2012) [25] using BSA substrate. Reaction mixture containing 0.5ml of enzyme solution and 0.5ml of 1%(w/v) BSA in 0.2M Phosphate buffer was incubated at 30°C for 10min. The enzyme reaction was stopped by adding 1ml of 10% Trichloroacetic acid containing 0.22M acetic acid and 0.33M sodium acetate. The reaction mixture was allowed to stand for 30 min at 30°C and then was filtered. 5ml of 0.55M sodium carbonate was added, followed by the addition of 1ml (3 times diluted) phenol reagent. The blue colour was measured at 660nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions.

Assay of Neutral protease

The Neutral protease activity in crude enzyme extract was assayed according to the modified method (Srividya shivakumar 2012) [25] using Casein as substrate. Reaction mixture containing 0.2ml of enzyme solution and 0.5ml of 1%(w/v) Casein in 50mM Phosphate buffer was incubated at 40°C for 20min. Except where specified, enzyme reaction was carried out at pH7.0. The enzyme reaction was stopped by adding 1ml of 10% Trichloroacetic acid. The reaction mixture was allowed to stand for 15 min at Room temperature. After centrifugation (10,000 rpm 5 min) and 2.5ml of 0.4M sodium carbonate was added, followed by the addition of 1ml of 3 times diluted phenol reagent and incubated at room temperature in dark 30 min. The blue colour was measured at 660nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions.

Assay of Alkaline protease

The alkaline protease activity in crude enzyme extract was assayed according to Srividya Shiva Kumar 2012 [25] using BSA substrate using casein as substrate. Reaction mixture of 1ml enzyme and 1ml of 0.5%(w/v) casein in 0.2M Tris-HCl buffer was incubated at 75°C for 1hour. Except where specified, enzyme reaction was carried out at pH7.4. The enzyme reaction was stopped by adding 2ml of 10% Trichloroacetic acid. The reaction mixture was centrifuged at 3000rpm for 10 minutes and supernatant was measured at 440nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions

RESULTS AND DISCUSSION:

Morphology of the selected mangrove plants

Rhizophora mucronata: The simple opposite, broadly elliptical, tooth-like leaf tip. Cuneate at base, leathery, leaf lower surface yellowish green, black dots scattered of the plant *Rhizophora annamaliana*: The leaves simple, opposite, broadly obovate, darkish green, leathery, black dots plenty.

Rhizophora appiculata: Simple opposite, narrowly elliptical, leathery midrib on lower part is pink, leaf tip acute. *Ceriops decandra:* simple opposite, obovete, leaf tip rounded, cuneate at base. *Suaeda monoica:* Alternately crowded, linear-oblong or spathulate, flat or sub terete, obtuse or rounded at tip, narrowed at base, black on drying.

ISOLATION OF ENDOPHYTIC BACTERIA:

Endophytic bacteria were isolated from different mangrove plants leaves. The isolated cultures were differentiated on the basis of their colony morphologies. A total of 44 bacteria isolates were finally selected, purified and maintained for further analysis.

CHARACTERIZATION OF IDENTIFICATION

Results of Gram straining test, Morphology test, Motility test, Spore test, Indole test, Methyl Red test, Voges-proskauer test, Citrate test, Catalase test, Oxidase test, Nitrate Reduction test, Urease test, Fermentation (Glucose) test, Fermentation (Lactose) test, Fermentation (Sucrose) test, H_2S production test are enlisted in Table 1. Of the 44 strains, based on the enzyme activities four strains were chosen for the further studies.

Table-1 Biochemical identification of the isolated Bacillus sp., bacterial strains

S.No.	Characters	*B.cereus*	*B.amyloliquefaciens*	*B.velezensis*	*B.siamesis*
1	Gram Straining	+	+	+	+
2	Morphology	Rod	Rod	Rod	Rod
3	Motility	+	+	+	+
4	Spore	+	-	+	+
5	Indole	-	-	-	-
6	Methyl Red	-	+	-	-
7	Voges-proskauer	-	+	-	-
8	Citrate test	+	+	+	+
9	Catalase test	+	+	+	+
10	Oxidase test	-	+	-	-
11	Nitrate Reduction test	-	+	-	-
12	Urease	-	-	-	-
13	Fermentation (Glucose)	+	+	+	+
14	Fermentation (Lactose)	+	+	+	+
15	Fermentation (Sucrose)	+	+	+	+
16	H_2S Production	-	-	-	-

Symbols were means (-) negative, (+) positive

All the strains were Gram positive, aerobic, motile, rod shaped, endospore forming bacteria. From the results of the biochemical tests it is confirmed that the species of interest could be of Bacillus sp. Bacillus sp. is one of the very common inhabitants of coastal and marine environment. Ammini Parvathi et al (2009) [26] .Agata Goryluk et al., 2009[27] isolated bacillus sp. from western coast of India from different sources.

MOLECULAR IDENTIFICATION

Molecular Taxonomy

The PCR product was sequenced and its 16S rRNA sequence was deposited in NCBI with accession numbers.

strain	NCBI accession number
BWC B19	9TVWM7K7014,
BWC BC.dec	EJKBX6VKO15.
BWC B14	EJKTKIED015,
BWC B18	EJKREBZ901R,

In the present study it was confirmed that all the four strains were of Bacillus species. BWC C. dec3- *Bacillus cereus,* BWC B14- *Bacillus amyloliquefaciens,* BWC B18- *Bacillus velezensis,* BWC B19- *Bacillus siamensis*. Ammini parvathi et al (2009) [28] have reported endophytic bacteria *B. cereus, B. amyloliquefaciens* and *B. subtilis* from the mangrove plant R. apiculata. Subramanian Deivanai et al (2014) [28] reported that B.pumilus were dominant to B.cerus, and B.sohaericus. Ivanova et al (1999) [29] also reported that B. pumilius and B.subtilis were dominant and was associated with marine sponges, coral etc.,

SEM ANALYSIS

Bacillus cereus: B.cereus is gram positive rod shaped bacillus or appears in short chains, non-capsulated, motile and flagellated. *Bacillus amyloliquefaciens* : Gram positive, motile rods, grouped in chain, endospores present. *Bacillus velezensis*: gram positive, motile, rod shaped. *Bacillus siamensis:* gram-positive, motile, rods,

GROWTH MEDIA

The mangrove plants isolated different source of nutrient agar medium. From the mangrove leaf sample 44 bacteria strains were isolated. But later during screening it was that only 4 strains showed enzyme production and qualitative and quantitative analysis.

The bacteria isolated from soil were screened for different medium growth 7 bacterial strains were isolated (Shyam sunder Alariya et al., 2013) [30].

ENZYME ACTIVITY

In the present study, 4 bacteria isolates showed the evaluated enzymes, 4 bacterial isolated enzymes proving their potential for industrial application. The enzymatic index was a fast and practical tool for selecting and comparing the enzyme production of different bacterial isolates. (Carrim et al., 2006) [18].

In further another point that should be explored is that mangrove microorganism demonstrates a diverse range of enzymatic activities and are capable of catalyzing various biochemical reactions using novel enzyme (Thatoi et al., 2013; Dias et al., 2009) [31][32]. Halophilic microorganisms in particular possess many hydrolytic enzymes and are capable of functioning under condition that lead to precipitation of denaturation of most proteins **(**Ventosa and Nieto1995) [33].

QUALITATIVE METHOD OF ENZYME ACTIVITY

The enzymatic index for all isolated was obtained following observation of their ability to produce at evaluated enzymes. Amylase 65%of reacted enzymatic activity and Gelatinase 5%, Keatinase 35 %, Chitinase 10%, protiase20%, Cellulae40%, pectinase55%, catalase70 %, Ligninase5% and overall high enzymatic reaction Amylase and Catalase for 70% compare with other.

bacterial community from mangrove sediments in Brazil had predominance of organisms from the orders *Vibrionales* and *Bacillus*. These isolates were also able to produce diverse extracellular enzymes such as amylase, protease, esterases and lipases, similar to the present work. The *Bacillus* spp. Strains from mangrove sediments reached 5.53 and 5.06 amylase and protease enzymatic indexes, our *Bacillus* spp. Strain reached 2.75 enzymatic indexes to both enzymes. However, the *Bacillus* sp.shows a lipase enzymatic index of 4.8, higher than of the best lipase producer *Bacillus* sp. 1A339 strain from mangrove sediment (Dias et al.2009) [32].

The Enzymatic Index for all isolated was obtained following observation of their ability to produce at least 1 to 5 evaluated enzymes. Amylolytic activity was observed in 45%, esterasic activity in 17.5%, lipolytic activity in 52.5%, proteolytic activity in

75% and cellulolytic activity in 62.5% of the tested isolated **(**Renata A Castro et al.,2004) [34]**.**

QUANTITATIVE METHOD OF ENZYME ACTIVITY

Quantitative method of Amylase activity

The present study Amylolytic enzyme production of *Bacillus* sp gradually initiated at 24 h of growth. Maximum amylolytic activity was observed at 24h of growth and it remains stable after Amylolytic activity of an endophytic bacterium (Fig 4).

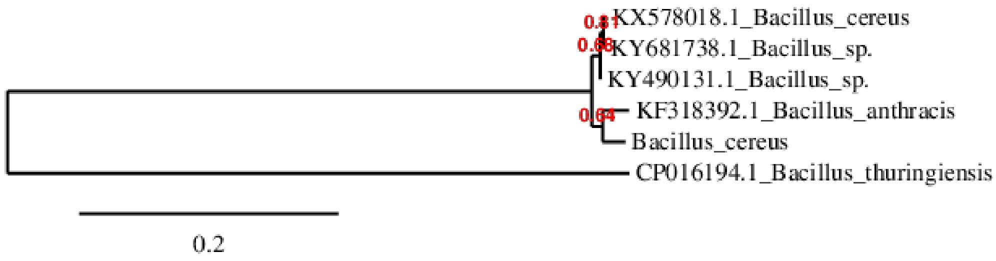
The result of enzymatic activity was isolated 6 bacteria tested are shown amylolytic activity 60% of enzyme activity is present (Aysha Jussara Ivoniled Carrim et al., 2006) [18]**.**

QUANTITATIVE METHOD OF CELLULASE ACTIVITY

In the present study, cellulolytic enzyme production of *Bacillus* sp gradually increased till 24 h. Maximum cellulase enzyme production was attained at 24 hours. After which it remained stable. (Fig 2).

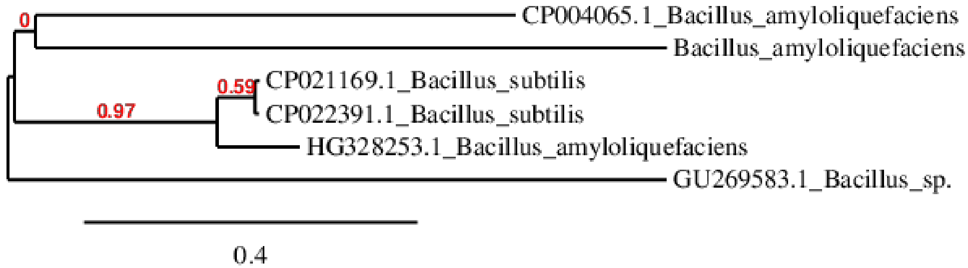
Quantitative method of Protease activity

In the present study, proteolytic enzyme production of *Bacillus* sp gradually increased till 24 h. Maximum prtoeolytic enzyme production was attained at 24 hours. After which it remained stable. (Fig 2).

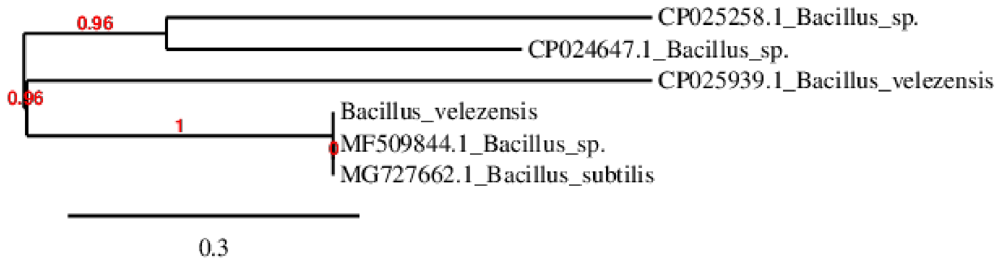


(Fig 1-A) Phylogenetic tree of: *Bacillus cereus*

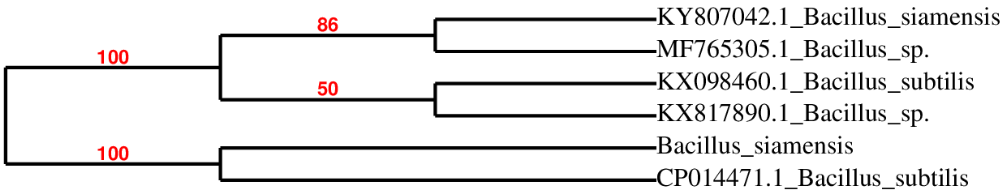
Phylogenetic tree of: Bacillus *amyloliquefaciens*



(Fig 1-B) Phylogenetic tree of *Bacillus amyoliquefaciens*



(Fig 1-C) Phylogenetic tree of *Bacillus velezensis*



(Fig 1-D) Phylogenetic tree of *Bacillus siamensis*

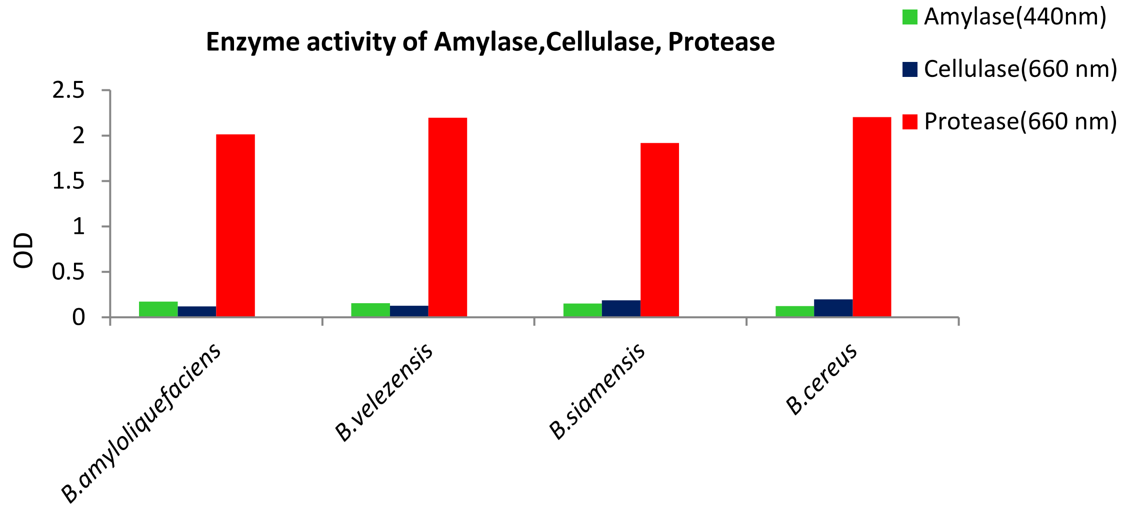


Fig2 Enzyme activity of Amylase, Cellulase, Protease

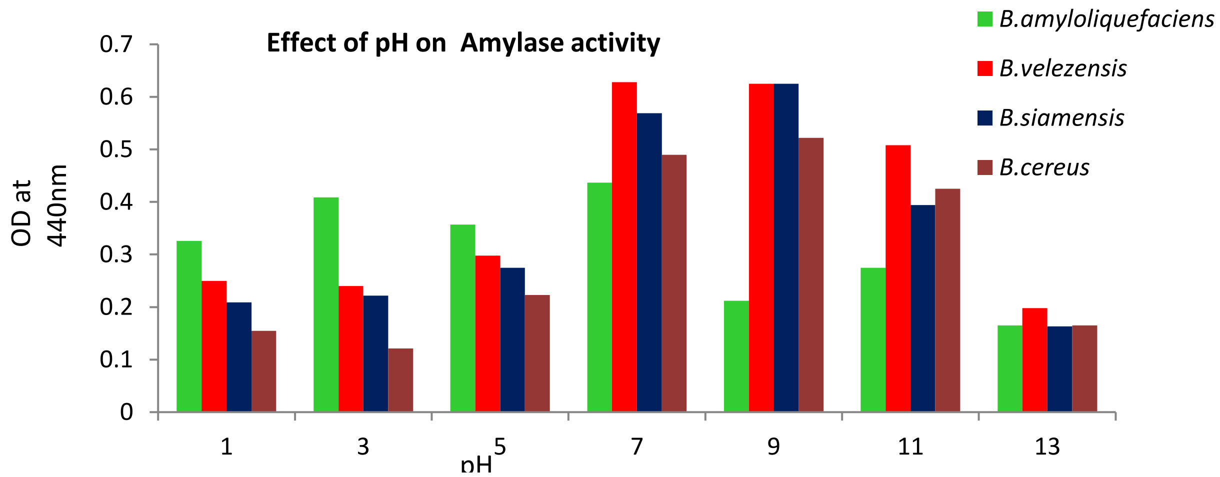


Fig3-a Effect of pH Amylase

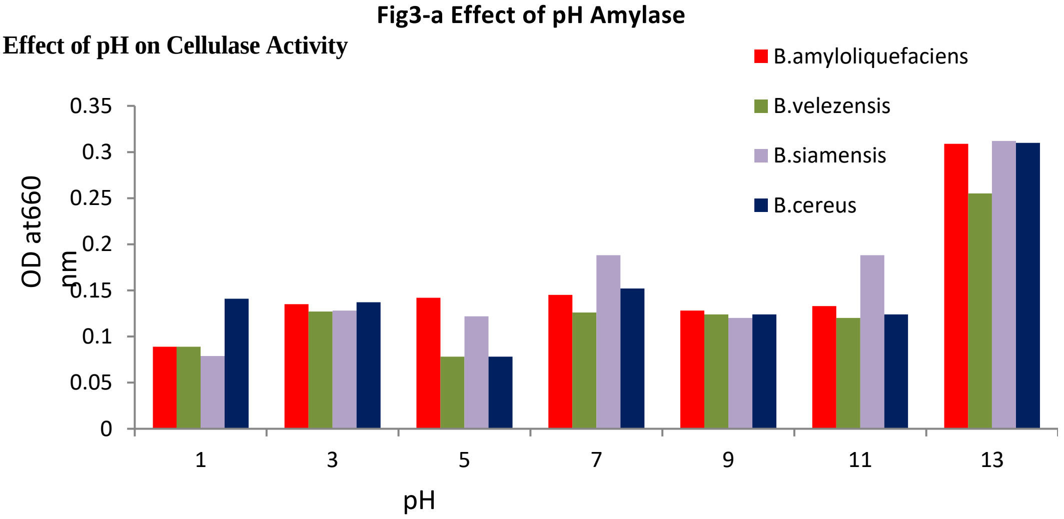


Fig 3-b Effect of pH Cellulase

Effect of pH on protease activity

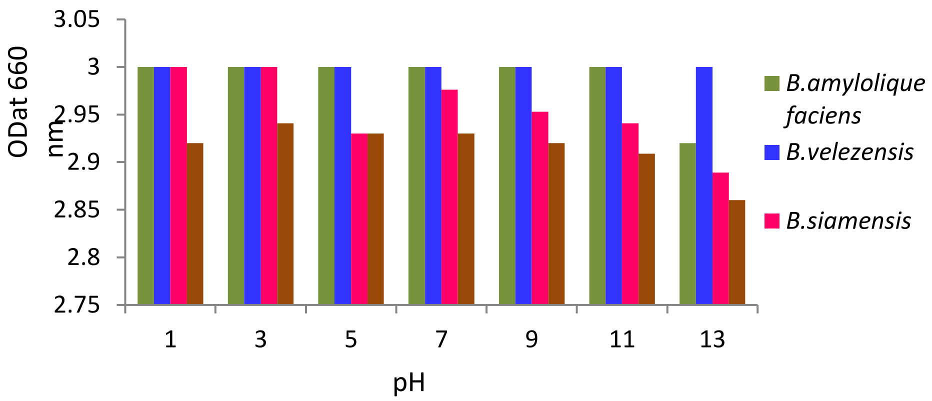


Fig 3-c Effect of pH protease

Effect of Temperature on amylase activity

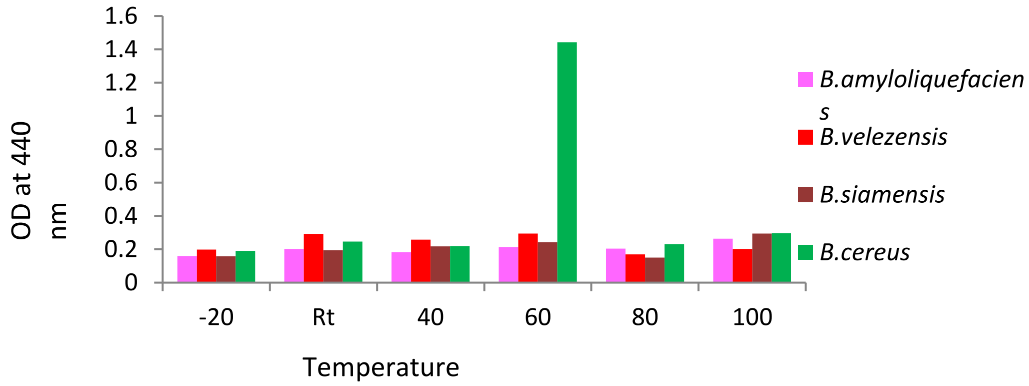


Fig 4-a Effect of Temperature on Amylase: -20- -20˚C, RT- Room temperature, 0-0˚C, 60-60˚C,80-80˚C,100-100˚C.

Effect of Temperature on Cellulase activity

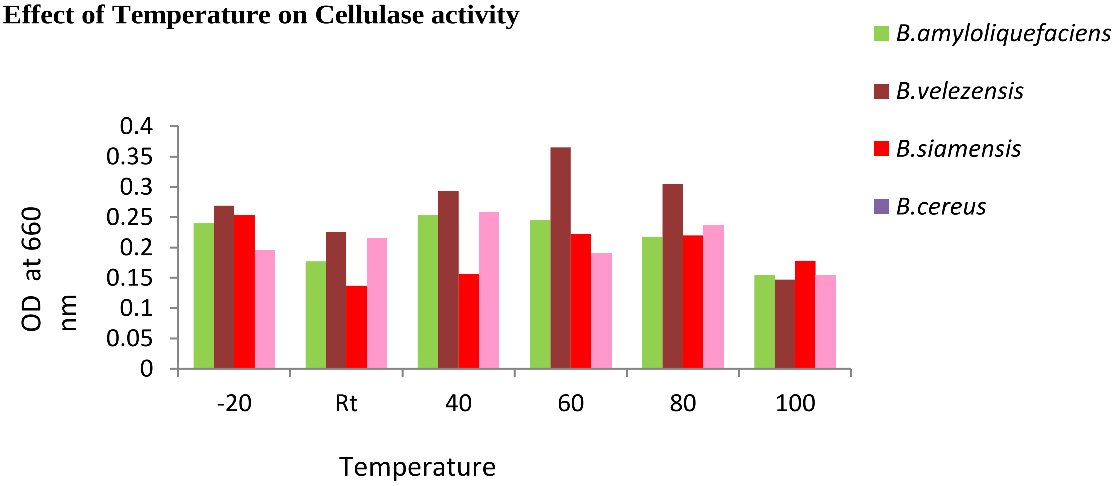


Fig 4-b Effect of Temperature on Cellulase: - -20- -20˚C, RT- Room temperature, 0-0˚C, 60-60˚C,80-80˚C,100-100˚C.

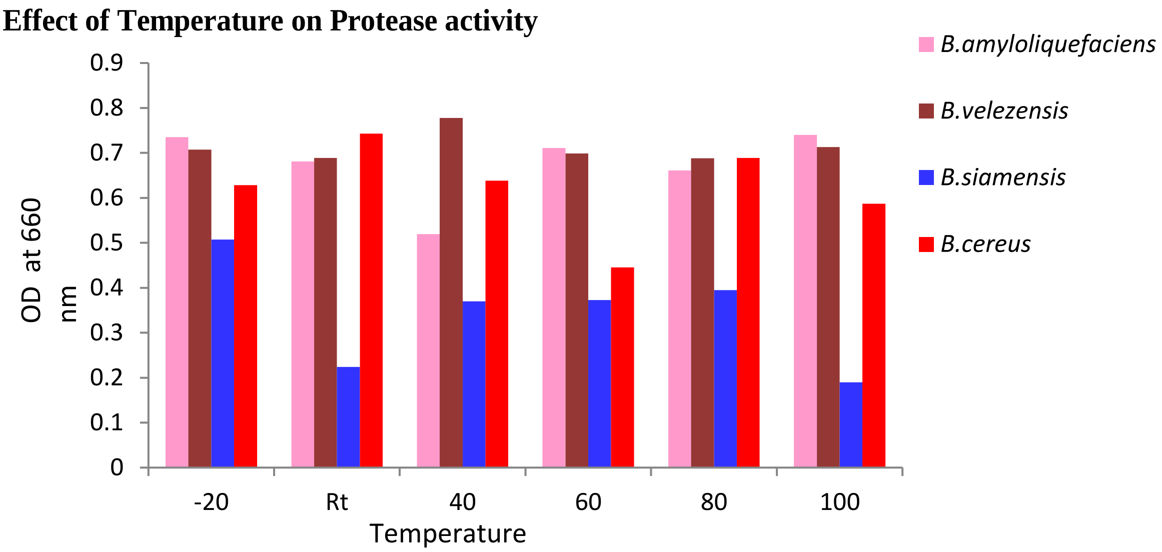


Fig 4-c Effect of Temperature Protease: - -20- -20˚C, RT- Room temperature, 0-0˚C, 60-60˚C,80-80˚C,100-100˚C.

EFFECT OF pH

Effect of pH with respect to protease production was carried out ie.acid, alkaline and neutral protease.(Fig 3-a, and c) Behal et al.,2006 [36] reported high alkaline protease production in *Bacillus* sp. in contradiction, in our studies we found *Bacillus* sp. to produce more of acid proteases.

EFFECT OF TEMPERATURE

Mohamed et al.,2009 [37] reported higher enzymes production at 35-40˚C thermal stability of bacteria. While in our case the thermal stability of bacterial enzymes was 60-80˚C. (Fig 4-a, b, c)

ACID, ALKALINE AND NEUTRAL PROTEASE

Proteases produced by the *Bacillus* sp. was active in pH ranges 1-13. It was positive for acid, alkaline and neutral proteases. However

The enzyme was stable in the temperature range of -20 to 100˚C with optimum temperature 80˚C. Acid protease was highly stable when compared with other neutral and alkaline protease. (Table 2). Francisco Javier Castillo-Yanez et al., 2003 [38] reported maximum proteolytic enzyme activity at pH 3.0.

B.subtilis different range of pH were used (9.2, 9.4, 9.6, 9.8 10, 10.2 and 10.4) the maximum result alkaline protease production O P verma et al (2011)[39].

Table 2: acid protease, neutral protease, alkaline protease

S.No	Name of Species	Acid protease	Neutral protease	Alkaline protease
1	*B.amyloliquefaciens*	4.000	4.000	1.329
2	*B.velezensis*	3.792	4.000	1.527
3	*B.siamensis*	4.000	4.000	1.308
4	*B.cereus*	3.505	4.000	1.272

CONCLUSIONS

44 Endophytic bacteria were isolated from mangrove leaves of *R.appiculata, R.annamalayana, R.mucronata, S.monoica* and *C.decandra.* Of which only 4 bacteria strain was potential enough to produce enzymes of interest. These bacteria were characterised and identified using 16 sRNA analysis and the deposited in NCBI.

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