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Regular Article

Bacillus isolates VTGP. A-D. 30808 *Alcaligenes sp., Exiguobacterium sp., B. pumilus* and *B. fusiformis* producing extracellular alkaline proteases, amylases and cellulases - a preliminary report

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Garden soil samples collected from Angamali, Kerala, India were screened for potent bacteria capable of synthesizing extracellular hydrolytic enzymes. Four bacteria were obtained in pure culture. The isolates were systematically identified by microscopy, Gram and special staining techniques for capsule and spores, biochemical reactions and phylogeny by molecular techniques like 16 S rRNA gene sequencing followed by Blast analysis. Production of protease, cellulase and amylase were detected by inoculating nutrient agar containing casein/ skim milk agar, carboxy methyl cellulose and soluble starch respectively. Alkalophilic and thermophilic properties were investigated by inoculation and incubation of the isolates on specific nutrient media at pH 7-12 and at a wide range of temperatures 28-30, 37, 50 and 65^0.C. The isolates were coded VTGP. A-D 30808. All the four expressed significant alkalophilic growth at pH 7-12. With respect to protease activity all except A showed marked protease activity over a high pH range pH 7-12 (A-115, B-1119, C-1500, D-1350 Units / ml of liquid culture supernatant). Both C & D secreted protease as early as 8-12 hours on nutrient agar with 0.1% skim milk forming a clear wide zone of casein hydrolysis. Hence the proteases produced were highly alkalophilic. Amylase activity was marked in all (A-37.38, B-27.58, C-27.92, D-34.82 units per ml culture supernatant). On CMC agar, all the four isolates showed CMCase activity indicated by pale yellow zone of hydrolysis of carboxy methyl cellulose agar when tested with Congo red reagent. A, B and C were strongly positive with minimal visible activity in D. But when tested in CMC broth culture the activities were A-6.71, B-4.30, C-6.56 and D 0.58 units/ ml of culture supernatant). 16S r RNA gene sequencing of isolates A to D showed maximum alignment with *Alcaligenes sp., Exiguobacterium sp., Bacillus pumilus* and *B. fusiformis.* The sequences have been deposited in GenBank with Accession numbers HQ 848384, HQ 848385, HQ 848386, and HQ 848387.

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Bacteria have been a rich source of a diverse group of primary metabolites. Among them bacterial enzymes consisting of cellulases, amylases, proteases and lipases constitute the most important hydrolytic enzymes with large scale application in industry. Proteases alone contribute to 60% of the global enzyme market (Debe *et al*., 2001; Kalisz, 1988; Kumar and Takagi, 1999; Outrup and Boyce, 1990; Rao *et al*., 1998). Most of the members employed in large scale enzyme production belong to genus Bacillus varying in their catalytic efficiency, substrate preference, specificity and physical parameters required for the diverse processes (Huang *et al*., 2004; Kim *et al*., 1997). The most common are *Bacillus cereus*, *B.subtilis, B.mycoides, B.circulans, B.sphaericus, B.coagulans, B.firmus, B.amyloliquefaciens, B.licheniformis* and *Clostridia.* With respect to cellulases and CMCases the Fungi and anaerobic bacteria occupy the frontline in industrial use. (Huang and Monk, 2004; Bhat and Bhat, 1997). The present work is an attempt in isolation and basic characterization of four aerobic or facultative anaerobic bacteria producing alkaline protease, amylase and cellulase (CMCase).

Materials and Methods:

Isolation and screening: Garden soil samples were collected from Angamali, Kerala and suspended in sterile saline. Aliquots were inoculated on nutrient agar plates. The plates were incubated at 37^0 C for 24 hours. The colonies showing clear difference in cultural characters were further purified by sub culturing on nutrient agar. The isolates were coded as VTGP.A-D30808 and preserved on nutrient agar slopes in the refrigerator.

Microscopic morphology:

Cell morphology, motility, Gram reaction, presence of spores and capsule and arrangement were studied using standard protocols.

Biochemical reactions:

Single isolated colonies from nutrient agar plates were tested for catalase, oxidase, inoculated into a variety of media for utilization of sugars, growth at low and high pH in peptone water. Further tests viz. salinity tolerance in 1-10% sodium chloride NA, growth at pH 7.0-12.0, ambient to 65^0 C on NA, citrate utilization, indole & urease production, MR,VP, Nitrate reduction, starch hydrolysis and gelatin liquefaction were tested on the best isolates. Results were analyzed as per Bergey's Manual of Systematic Bacteriology (Holding & Shewan, 1974)

Enzyme production:

Screening for extra cellular hydrolytic enzymes was done by inoculating each of the four isolates on special media such as nutrient agar containing specific substrates like starch, casein and carboxy methyl cellulose at 1, 0.1 and 0.5% respectively. The plates were incubated at 37^oC. All enzyme activities were tested as per standard protocols. Amylase secretion was tested by covering the plates with iodine potassium iodide reagent and observation of clear zones (Holding & Shewan, 1974). Proteolytic activity was measured by the clearance

indicating hydrolysis of casein (Berla Thangam & Suseela Rajkumar, 2000). CMCase activity was determined by flooding the plates with Congo red followed by saturated sodium chloride solution (Huang and Monk, 2004).

Enzyme activity in liquid media:

Four sets of Nutrient broth 20 ml each containing 1% starch pH 7.0, 0.1 % casein pH 8.0 and 0.5% carboxy methyl cellulose pH 7.0 were inoculated with each isolate from fresh agar slopes and incubated for 24 hours. The broth was centrifuged at 6000 rpm for 20 minutes at 20 deg C. Supernatant was transferred to the refrigerator. The culture filtrates were analyzed for pH, total soluble proteins (Lowry, 1951) and all enzyme activities. Protease activity was measured as described elsewhere with slight modifications using 50 mM Tris Hcl buffer pH 8.5 and casein as substrate (Berla Thangam & Suseela Rajkumar, 2000). Amylase was estimated as per standard protocol using soluble starch as substrate in 0.1 M phosphate buffer pH 7.0 (Ajayi, 2007)**.** CMCase was tested with carboxy methyl cellulose in citrate buffer pH 6.5 as per Seimon *et al* (2006). Enzyme substrate mixtures were incubated in water bath at 37 deg C for protease and 50 deg. C for both amylase and CMCase activities. Appropriate controls were included for all assays.

Effect of medium pH on protease production:

Each of the isolates A, B, C and D was inoculated on NA containing skim milk 0.1% pH 7,8,9,10,11 and 12. They were incubated at 37 deg C for 24 to 48 hours. The growth and zone of clearance were noted and compared for each isolate at different pH.

Effect of temperature on growth and protease production:

Plain Nutrient agar slopes and Casein Nutrient agar plates were inoculated with A, B, C and D cultures and incubated at 37, 50, 65^0 C. Density of biomass and zone of clearance were observed.

Molecular identification of species by 16 S r-RNA gene sequencing:

This was carried out by standard protocols as follows (Chromous services Chennai)

1. **Genomic DNA was isolated** from the pure culture pellet (Chromous Genomic DNA isolation kit RKT09) and loaded on 1 % agarose gel. Standard used was 1 kb DNA Ladder (Chromous Cat. No. LAD03).The ~1.4 kb rDNA fragment was amplified using high –fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse Primer- 16s rDNA primers. The PCR product was gel eluted and the purified PCR product was sequenced using 2 primers.

2. PCR Amplification conditions:

DNA: 1 µl; 16s Forward Primer 400 ng; 16s Reverse Primer 400ng; dNTPs (2.5 mM each) 4 µl; 10 X Taq DNA Polymerase Assay Buffer 10 µl; Taq DNA Polymerase Enzyme (3U/ µl) 1 µl; Water X µl; Total reaction volume: 100 µl. All PCR reagents were of Chromous Make.

Profile: Initial denaturation: 94 for 5 min; Denaturation: 94 for 30 sec; Annealing: 55 for 30 sec Extension: 72 for 2 min; Final extension: 72 for 15 min; Mgcl2: 1.5mM final conc.
Number of Cycles: 35

3. Forward and reverse primer sequence which was used for amplification of 16s rDNA sequence: Prokaryotes: 16s rRNA specific primer
Forward Primer: 5'-AGAGTRTGATCMT YGCTWAC-3';
Reverse Primer: 5'-CGYTAMCTTWTT ACGRCT-3'

Results and Discussion

Results are shown in Fig. 1 - 17 & Table I
Isolation of bacteria, microscopy and cultural characters: Soil samples inoculated on basal nutrient agar showed four clearly different morphological types of colonies. They were coded A, B, C and D.

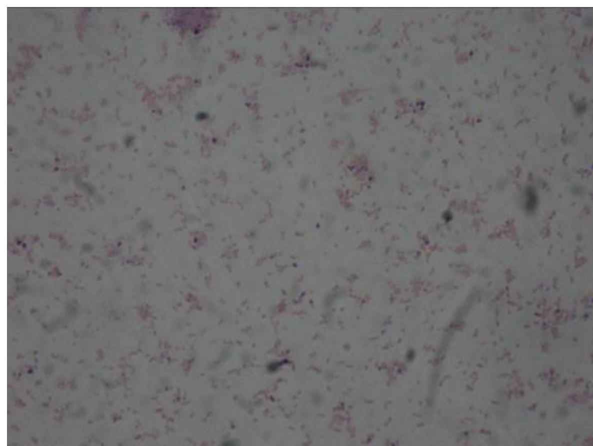

Fig1. VTGP.A30808 Gram stains at 100 X

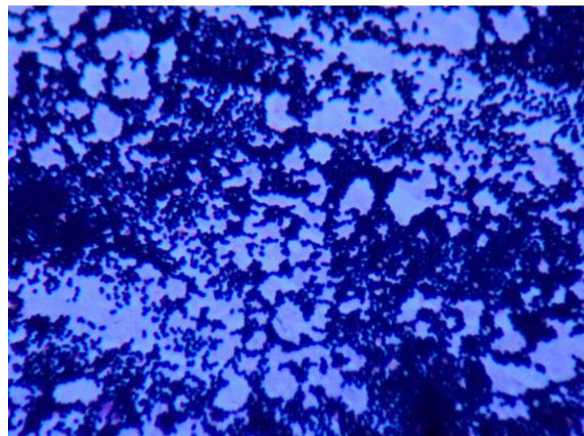

Fig 2. VTGP.B30808 Gram stain at 100 X

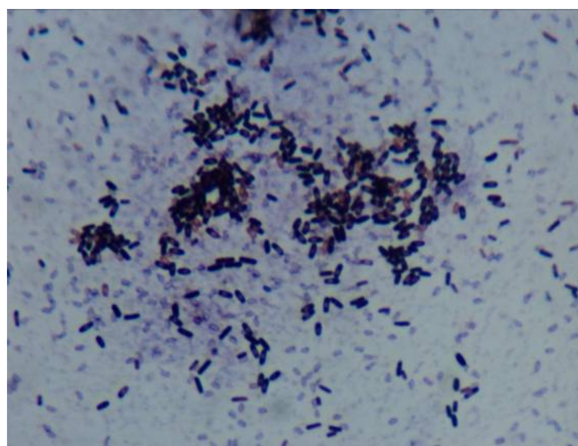

Fig 3. VTGP.C30808 Gram stain at 100 X

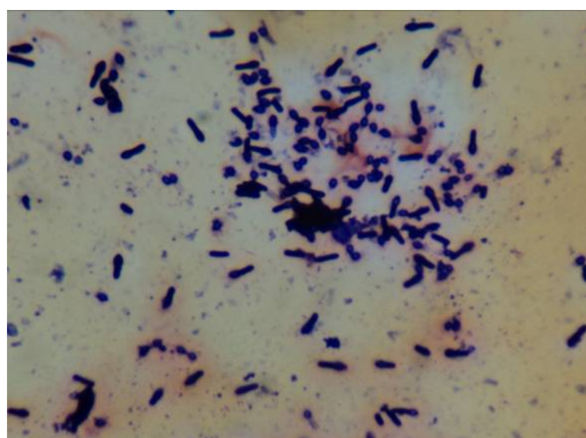

Fig 4. VTGP.D30808 Gram stain at 100 X

Colony A: Gram showed short slender highly coco bacillary Gram negative rods, non sporing, arranged in singles, non capsulate, sluggishly motile. At 24 hours at 37 deg.C colony was about 1-2 mm diameter, pale, thin, transparent, very moist, flat, non pigmented and easily emulsifiable. It was catalase positive, oxidase positive. It was a facultative anaerobe, growing better in aerobic condition, Glucose fermentation was negative, no growth in anaerobic glucose peptone water, and grew on Mac Conkey's agar. pH range was wide from 7-12. Starch hydrolysis was positive. CMCase activity was strongly positive, protease activity nil in pH 7-12

Colony B: Gram positive, thick, ovoid, capsulate, arranged in singles and very short chains, non sporing, motile, bulging the cell wall, stout and spindle shaped. On nutrient agar it showed orange colored colonies, large, 3-4 mm diameter, opaque, mucoid, margin irregular and spreading. Biochemically starch hydrolysis was positive, nitrate reduction negative with good growth on nutrient agar on a wide range of pH from7.0 -12.0. Amylase, protease and CMCase activities were strongly positive.

Colony C: Gram staining showed Gram positive bacillus, thick, large central prominent spores, non bulging, ends rounded, non capsulate, in singles. It was motile, catalase positive, oxidase negative. Colonies on nutrient agar incubated at 37 degree C for 18 hours were small circular, white, mucoid, spreading edges, flat, moist and easily emulsifiable. Very minimal growth but marked protease activity was observed even within 12 hours at 50 deg C. Growth was poor at 50 while totally nil at 65 deg C. Starch hydrolysis and CMCase were strongly positive over pH range 7.0 to 12.0. Protease activity was high with the isolate producing a wide zone of hydrolysis in skim milk agar at pH 7.0-12.0. The optimum temperature for proteolysis on solid growth medium was 37^0 C. The organism was not salinity tolerant and was inhibited by 2% sodium chloride. Thus C was found to be a highly alkalophilic, proteolytic, amylolytic and CMC degrading species. Gelatin liquefaction was positive. There was no growth on MacConkey, fermented glucose, maltose, sucrose and mannitol, lactose negative , MR positive, VP negative, Indole and citrate negative (Table 1, Fig.5-8).

Colony D: Gram showed strongly Gram positive, large, 3-5 microns size, with prominent terminal spores, bulging, and club shaped cells in singles. The isolate was motile. On NA it formed pale pink colonies, moist, flat, easily emulsifiable, edges irregular, catalase positive and oxidase negative. There was strong starch hydrolysis, and a wide zone of hydrolysis on skim milk agar over pH 7.0-12.0. CMCase was negative on CMC agar.

Fig.5 VTGPC.30808 Catalase +

Fig 6: . VTGP.C30808 Gelatin liquefaction

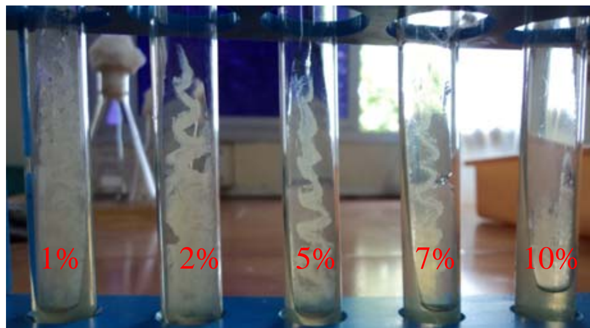

Fig.7. VTGP.C30808- **Growth on NaCl - NA**

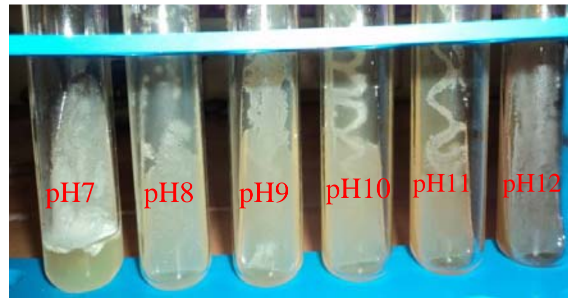

Fig. 8 VTGP.C30808 Growth on NA pH 7-12

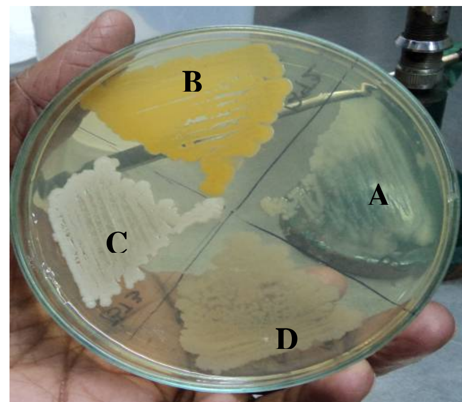

Fig.9 VTGP. A-D.30808: Colony characters on NA

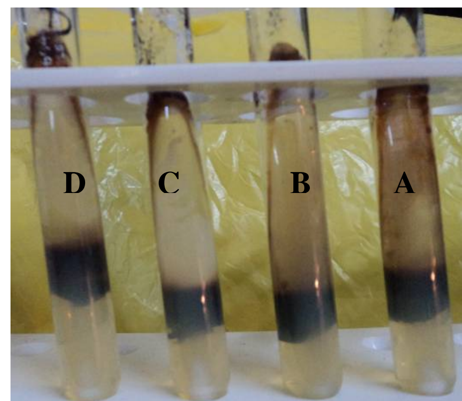

Fig.10 Amylase activity on starch

Fig.11 Protease activity on skim milk agar at 12h

Fig.14 Growth at 37 ° C 12 hours

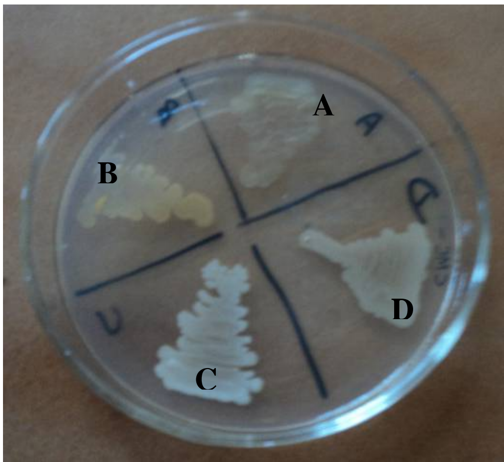

Fig.12 Growth on CMC agar

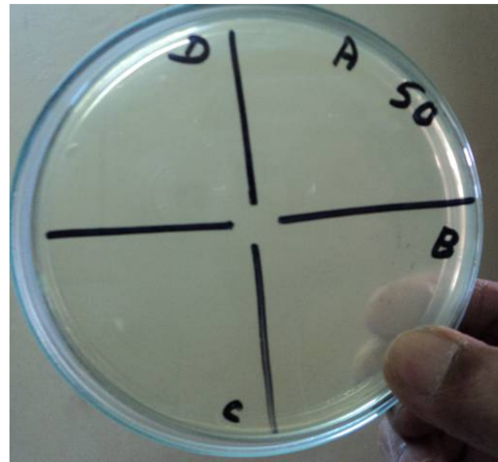

Fig.15 Growth at 50^{0}C on skim milk agar

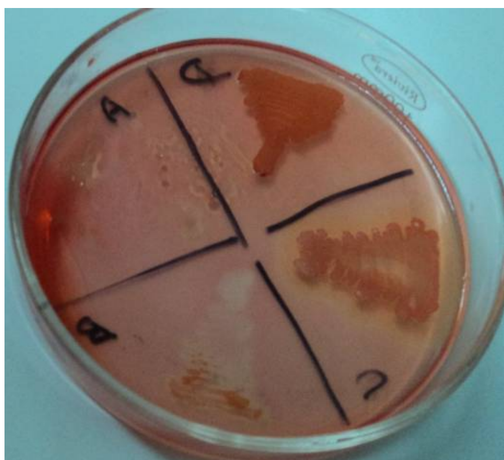

Fig.13 CMCase +A, B&C Congo red

Fig. 16- Total casein hydrolysis at pH 11

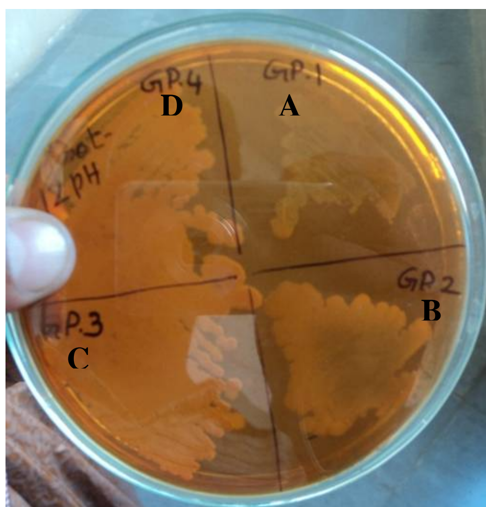

Fig. 17. Total casein hydrolysis at - pH 12

The isolate VTGP.A30808 identified as *Alcaligenes sp.*, produced high levels (37.38 Units) of amylase activity compared to 115 units of protease and 6.71 units of CMCase per ml of culture supernatant. There are very few reports available on amylases and cellulases from *Alcaligenes sp.* Culture supernatants of *Alcaligenes faecalis* showed maltotetraose-forming amylase (Zhu *et al*, 1997). *Alcaligenes faecalis* has however been shown to produce high levels of extra cellular proteases (171.2 U) in alkaline pH-8.0 (Berla Thangam & Suseela Rajkumar, 2000). Our isolate seems to be an unusual strain with a significant potential for amylase enzyme secretion. *Exiguobacterium sp.*, isolated in the present work was also highly alkalophilic growing luxuriously over pH 7.0 to12.0 with 1119 units/ml of protease, 27.58 units/ml of amylase and 4.30 units /ml of cellulase (CMCase) enzyme. Achal *et al* demonstrated *Exiguobacterium* isolated from adverse and extreme environmental conditions such as cement samples with

Table 1. VTGP. C 30808 microscopic morphology and biochemical properties

Parameters		Result
Gram		GPR
Motility		+
Catalase		+
pH	>=6.8	+
	<= 5.7	+
Temperature	37°C	+++
	40°C	++
	50°C	+
	60°C	-
	65°C	-
	70°C	-
	80°C	-
Salinity	1%	++++
	2%	+++
	5%	++
	7%	+
	10%	-
Starch hydrolysis		+
Casein hydrolysis		+
Growth at pH	7	+++
	8	+++
	9	+++
	10	+++
	11	+++
	12	+++
Gelatin liquefaction		++
Growth	nutrient agar	+
	Mac Conkey	-
Carbohydrate fermentation	Glucose	A
	Sucrose	A
	Maltose	A
	Mannitol	A
	Lactose	-
	Xylose	-
	Arabinose	-
IMViC	Indole	-
	Methyl red	+
	Voges proskeur	-
	Citrate	-

high alkalinity pH 6.0-12.0 and growing in high salinity, 10% sodium chloride medium producing alkaline protease at 575.8 units/ml of culture filtrate. The protease production was maximum at pH 10.0 by their isolate *Exiguobacterium sp* YS 1 (Achal *et al*., 2010). Many members of *Exiguobacterium* have been reported to be highly alkalophilic (Suga and Koyama, 2000) producing alkaline proteases (Ramesh *et al*., 2007) and alkaline esterase (Hwang *et al*., 2005). A psychrotrophic alkali tolerant *Exiguobacterim sp* was isolated from extreme cold (5^0C) western Himalayas producing alkaline protease at 1.46 units/mg protein, stable at 50^0C with optimum pH 8.0. The *Bacillus pumilus* isolated by us was an extremely powerful producer of all three enzymes at ambient and 37°C. It could secrete alkaline protease as early as 12 hours on skim milk agar. In skim milk broth it showed the highest protease activity 1500 units/ml; and others enzymes being amylase 27.92 units/ml and CMCase 6.56 units/ml. The organism has been unique. Similar results with respect to alkalophilic/ alkali stable protease synthesis has been reported by other workers. An alkaline Mn^{++} dependent serine protease from *B. pumilus* TMS55 was purified and characterized with optimum pH7-12 and activity of 5.8 units/ml (Ibrahim *et al*., 2011). Cellulase and CMCase production by *B. pumilus* in particular have been reported by another group of workers (Simeon *et al*., 2006). The CMCase was active at 40 - 65^0C and pH 6.5 - 7.Amylase production by *B. fusiformis* has not been reported so far as per available literature. *Bacillus fusiformis* in our study showed again high protease 1350 units/ml at high pH 7 - 12 as well as amylase 34.82, and very minimal CMCase 0.58 units/ml in comparison with others.

The 16S rRNA gene sequencing and sequence data analysis could assign the isolates to Genus *Alcaligenes sp., Exiguobacterium sp., Bacillus pumilus* and *Bacillus fusiformis*.

Conclusion

The present work reports isolation, biochemical and phylogenic identification, and, basic characterization of four highly potent uncommon bacteria producing a variety of extra cellular hydrolytic enzymes in significant amounts. The isolates were identified as *Alcaligenes sp, Exiguobacterium sp, Bacillus pumilus* and *B. fusiformis* with Accession numbers GenBank HQ 848383 – HQ 848387. Among the isolates *Exiguo-bacterium sp., B.pumilus* and *B. fusiformis* produced highly alkali stable proteases over a medium pH 7.0 - 12.0. All secreted significant amounts of amylases and CMCases also. The isolates are unusual in both occurrence as well as enzymatic potential. The most notable finding is amylase production by *Bacillus fusiformis*; growth of all isolates over a high pH up to 12.0 and production of protease, amylases and CMCases in very significant degrees by both *Exiguo-bacterium sp.* and *Bacillus pumilus*. The isolates hold enormous prospects for more extensive molecular characterization and application in a wide range of industries from food, textile, confectionary, environmental, tannery, detergent, pharmaceutical, medical

biotechnology as well as molecular biology and genetic engineering.

Acknowledgement

I dedicate the isolates to my Advocate **G**eorge **P**oonthottam, High Court, Kerala

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