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Molecuar Characterization And Optimization Of Cellulase Producing Bacteria From Fruit & Vegetable Peel Wastes As A Substrate

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ABSTRACT

The aim of the present study was planned to isolate, identify, screening and molecular characterization of bacteria with high cellulase activity from fruit wastes and optimization of cellulase production. In the present study cellulase producing bacteria were isolated from fruit wastes on screening media containing Carboxy Methyl Cellulose (CMC). The organisms were identified using morphological and biochemical characterization. The two strains like Pseudomonas aeroginosa and Bacillus clausii were showed a sequence similarity upto 99% in 16S rRNA. Cellulase enzyme producing microorganism was isolated from fruit wastes and identified and molecularly characterised as Pseudomonas aeroginosa. The media was optimized for cellulase production and the isolateproduces maximum cellulase at pH 9.1, temperature 40 ºC and 72 hrs of incubation time. Cellulase characterization shows that, cellulase has optimum activity at pH 5, temperature 45 ºC and 15 min of incubation with CMC as substrate. The Pseudomonas aeroginosa, showed the 78.03% yield of cellulase. Among bacteria, Pseudomonas aeroginosa, is the best cellulose producer among the four followed by Bacillus clausii, Cellulomonassp, Escherichia coli, and Lactobacillus sp. Results shown that the bacterial isolates produced significant level of cellulase with cellulose degrading capability.

Keywords: ***Cellulase, waste fruit peel, vegetable peel materials, CMC, molecular characterization, fermentation***

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1. INTRODUCTION

Cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem and is the main component of plant biomass (Shankar et al., 2011). It is the dominant waste material from agricultural industry in the form of stalks, stems and husk, there has been great interest in utilizing cellulose as an energy resource and feed (Balachandrababu et al., 2012). The cellulose is composed of D-glucose units linked together to form linear chain via ß-1,4-glycosidic linkages.

Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. The complete degradation of cellulose is made by a cellulolytic enzyme system. It has been established that there are three main types of enzymes found in the cellulase system that can degrade cellulose: exo- 1, 4-glucanase, endo- -1, 4-glucanase and -glucosidase. The endoglucanases act internally on the chain of cellulose cleaving -linked bond liberating non reducing ends, and exoglucanase remove cellobiose from this non-reducing end of cellulose chain. [Sun and chang,2002].

Bioconversion technology is preferred for raw materials with uniform composition and low lignin content. Such raw materials include agricultural residues such as crop waste (rice straw, wheat straw and plant stalk), wood and grasses (switchgrass). In this process, microbial enzymes perform the three processes such as lignin breakage, cellulose hydrolysis and fermentation. Currently, bioconversion process is applied for ethanol production using commercial lignase and cellulase enzymes.

2. MATERIALS AND METHODS

Sources and isolation methods

Waste substrate samples such as Orange peel, Bamboo leaves, Pineapple peel, Banana peelBeet root peel were collected in sterile separate plastic covers from domestic area of Tiruchirappalli (10°48’03.40”N and 78°42’55.31”E)

Acid pre-treatment

The dilute sulfuric acid pre-treatment of fruit waste substrate was carried out using varied acid concentration (1-5% w/v) and incubation time (15-60 min) at 121°C. The

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hydrolysate after treatment was separated by filtering the contents through double layered muslin cloth. The residual biomass was washed with tap water till neutral pH and dried in a hot air oven at 60°C. The oven dried pre-treated material was then analysed.

Alkali pre-treatment

The substrate was presoaked in different concentration of alkali (NaOH) ranging from 1 – 5 % (w/v) for 2h and thereafter thermally pre-treated at 121°C for 15, 30, 45 and 60 min. The pre-treated material was filtered through double layered muslin cloth, washed extensively with tap water until neutral pH and dried at 60°C. The oven dried pre-treated material was then analysed.

Isolation

One gram of the sample was enriched in 100 ml of Berg's medium [containing (l-1): 2 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$, 0.05 g K_2HPO_4, 0.01 g $FeSO_4.7H_2O$, 0.02 g $CaCl_2$ and 0.02 g $MnSO_4$, pH 7.0] (Immanuel et al., 2006) and incubated at 37°C on a rotator shaker (200 rpm) for 2 days.

The 0.1 ml suspension of 10^6 dilution in sterile 0.85% NaCl solution was transferred to Carboxymethyl Cellulose (CMC) screening medium [contain (l-1): 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 1.0 g CMC and 15 g agar, pH 7.0] (Shankar et al., 2011) and incubated at 37°C for 2 days. The colonies were isolated and purified by streaking on CMC agar plate. The pure cultures were maintained on LB slant at 4 °C for further analysis.

Screening of cellulase activity

Bacterial isolates were individually inoculated on CMC agar plates and incubated for 2 days. The plates were flooded with 0.1% Congo red for 20 min and washed with 1 M NaCl for 15 min. The clear zone formed by the isolates was indicated their cellulase activity (Lisdiyanti et al., 2012).

Estimation of reducing sugar by dnsa method

The reducing sugar produced in the reaction mixture was determined by Dinitro-salicylic acid (DNS) method (Miller 1959). 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the colour developed was read at wavelength 540 nm by

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using the spectrophotometer. One unit of cellulose activity was defined as the amount of enzyme releasing 1 mole of reducing sugar /ml /h.

Calculation

Enzyme activity (U/ml) for CMCase activity and FPase activity = O.D at 540nm ×µM of glucose (from standard graph) ×dilution factor0.1 ×Incubation time (min) ×aliquots of enzyme (ml) ×M W of glucose (gm)

Identification methods phenotypic characterization

Cells grown on Luria-Bertani (LB) agar medium were examined for their morphological and cultural characteristics, including cell shape, colonial appearance, endospore formation and pigmentation, after incubated at 37°C for 2 days.

Catalase and oxidase, the methyl red (M R) and Voges-Proskauer (VP) reactions, indole production, H_2S production and acid formation from carbohydrates were determined as described by (Barrow and Feltham 1993).

Genotypic characterization

Purification of pcr production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Sequencing protocol Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Pcr protocol

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Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a singlestranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the taqmaster mix

• Taq DNA polymerase is supplied in 2X Taq buffer
• 0.4mM dNTPs,
• 3.2mM MgCl2 and
• 0.02% bromophenol blue.

Add 5 µL of isolated DNA in 20 µL of PCR reaction solution (1.5 µL of Forward Prime and Riverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

Bioinformatics protocol

1. The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007).Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of

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magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper et al.,2008).

Measurement of crude cellulase

The 2% inoculum of bacterial isolates were inoculated into production medium containing (g/L), 10g CMC, 0.2g MgSO4.7H2O, 0.75g KNO3, 0.5g K2HPO4, 0.02g FeSO4.7H2O, 0.04 CaCl2, 2 g yeast extract and 1g D-glucose, at pH 7.0 (Lisdiyanti et al., 2012) and incubated at 37°C on rotator shaker (200 rpm) for 2 days. After incubation, the fermentation broth was harvested by centrifugation at 5,000 g for 20 min at 4 °C. The cell free extract was used as crude enzyme to measure the cellulase activity. Cellulase activity was determined by estimating the reducing sugar produced during enzymatic reaction by dinitrosalicylic acid (DNS) (Miller, 1959).

Purification of enzymes

The cured enzyme was brought to 45% saturation with Ammonium Sulphate and kept overnight in cold room at 4ºC. It was there after subjected to centrifugation at 8000rpm for 10 min at 4ºC. The Precipitate was discarded, while the supernatant was brought to 85% saturation with ammonium sulphate and centrifuged at 8000rpm at 4ºC for 10min.The precipitate from this step was collected and stored at 4ºC. Dialysis tubes, which were previously soaked in 0.1M . Phosphate buffer, pH, 6.2, was used for the dialysis of precipitated. The precipitated was dissolved in 0.1M . Phosphate buffer and dialyzed against the same buffer. The enzyme further purified by passing through a column of activated DEAE –cellulose previously equilibrated with 0.1M Phosphate buffer, pH, 6.2. A total of 30 fractions were collected at the flow rate of 5 ml/30 min. (Muhammad et al., 2012).

Process optimization for maximum cellulase production

Media optimization:

The optimum conditions for cellulase production were determined for the selected isolates. The cellulase fermentation was carried out at different pH, temperature, and incubation time and the crude enzyme was collected from each set to check the enzyme activity. (Sonia Sethi,Aparna Datta., *et al.* (2013)

Effect of incubation period on cellulase production:

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Different incubation times (24, 48, 72 and 96 hours) were employed to study effect of time on cellulase production. The culture filtrates were collected at respective time interval and crude enzymes assay carried out using CMC as substrate at 45 ºC.

Effect of ph on cellulase production:

For determination of optimum pH for cellulase production, the isolate was inoculated in the production media of different pH ranging from 5, 6, 7, 8, 9, 10 &11 for 72 hrs at 37 ºC. The crude enzymes assay carried out using CMC as substrate at 45ºC.

Effect of temperature on cellulase production:

For the determination of optimum temperature for production of cellulase, the isolate was inoculated in production media and fermentation was carried out at various temperatures in the range of 5 ºC, 15 ºC, 25 ºC, 35 ºC, 45 ºC at pH 7 for 72 hrs. The crude enzymes assay was carried out using CMC as substrate at 45 ºC (Shaikh et al., 2010, Saraswati et al. 2012, and Abubakar et al., 2013).

Effect of carbon sources

The effect of various carbon sources such as starch, glucose, maltose, lactose, and fructose at the concentration of 1 to 5% was examined in the production medium.

Effect of nitrogen sources

Various nitrogen sources like yeast extract, urea, and ammonium sulphate were examined for their effect on enzyme production by replacing 0.5% peptone in the production medium.

3. RESULTS AND DISCUSSION

Cellulase-producing bacteria were isolated from fruit and vegetable peel wastes samples. Appropriate dilutions of each sample were inoculated on CMC agar plates. Using of CMC as the sole carbon source plays a pivotal role for achieving the highest level of cellulase production because CMC induces cellulase gene expression. There are five bacterial colonies were isolated with higher cellulase activity then it was further screened to obtain the pure culture. The pure culture produced clear zone when it was flooded with Congo red solution due to hydrolysis of CMC. Gram's iodine forms a reddish brown complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct clear zone around the cellulase-producing

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bacterial colonies within 3 to 5 minutes. Based on the morphological and biochemical characteristics and molecular characterstics, the isolates were identified as ***Pseudomonas aeroginosa, Bacillus clausii,Cellulomonassp, Escherichia coli, and Lactobacillus sp.***The bacterial cultures produced zones of hydrolysis in CMC agar plates within 3 days and results were represented. Among the five isolates, two isolates (*Pseudomonas aeroginosa, Bacillus clausii*) were showed an excellent cellulase hydrolysis capacity ranged from 2.76 to 5.34 IU/ml when screened by Congo red method.

Molecular characterization of cellulase enzymes producing bacteria

Two different maximum levels of cellulase enzymes producingbacterial strains such as were analyzed for molecular characterization.

Isolation of DNA

Thepurified three isolated bacterial genomic DNA were separated by agarose gel electrophoresis. The separated bacterial genomic DNA was used for further study. After separation, the result of DNA fragments are visible as clearly defined bands. The DNA standard should be separated to a degree that allows for the useful determination of the sizes of sample bands. In this study, DNA fragments ranging from 2361 bp to 2027 bp were separated on a 1.5% agarose gel.

Pcr amplification of 16s rRNA

Extracted genomic DNA containing 16S rRNA from each isolate was amplified for gene sequencing using PCR. Partial sequencing of the genomic DNA of the test isolates revealed that the 16S rRNA portion contained base pairs ranging from 1311 to 1456.

Nucleotide sequence accession numbers

The present study based on partial 16S rRNA sequencing of the sequences obtained from the effective isolates when subjected to BLAST. The isolate from orange peel showed sequence similarity of 99% with *Pseudomonas aeruginosa*. The isolatefrom bamboo showed sequence similarity of 99% with *Bacillus clausii*.The sequence has been deposited at GenBank Bethesda, MD, USA. The details regarding the number of base pairs, accession number as provided by GenBankfor the effective test isolates. After the alignment, the tree building option

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can be activated using Bioedit Software. The tree viewing software njplot is used to generate a cladogram the bacterial isolates as shown in the table 3.

Blast analysis of the 16S rRNA gene sequences of the isolates with the reference 16S rRNA gene sequences confirmed the identity of the isolates already made based on morphological and biochemical characterization. Based on the molecular characteristics and sequence alignments the isolated strains were conformed as *Pseudomonas aeruginosa* and *Bacillus clausii*

OPTIMIZATION OF CELLULOSE ENZYME

Effect of pH

All the five isolates were allowed to grow in media of different pH ranging from 6.0 to 11.0. Maximum enzyme activity was observed in medium of pH 9.0–11.0 in case of *Pseudomonas aeroginosa, Bacillus clausii, Cellulomonassp, Escherichia coli, and Lactobacillus sp*. **(P. Chantawannakul, 2002., A. M. Abdel 2008., W. Win., 2008)**

Effect of incubation temperature

Enzyme activity recorded at different temperatures revealed that all the five bacteria yielded maximum cellulase production at 40°C. The temperature was found to influence extracellularenzymesecretion,possiblybychangingthephysical properties of the cell membrane. Optimum temperature for maximumgrowthof*Pseudomonas aeroginosa*,was 40°C. And for the Bacilusclausii they have a optimum temperature at 38°C. these two organism that showsa the exact optimum temperatures **(E.Jansov´a, 1993. M. K. Bakare, 2005. A. K. Ray, 2007.,G.Immanual 2006)**

Effect of carbon source

Various sources of carbon such as starch, fructose, maltose, and sucrose were used to replace glucose which was the original carbon source in growth media. Results obtained showed that glucose brought the highest cellulase production compared to other carbon sourcesat24hincubation.Lactoseandfructosealsoshowed high cellulase production at 24h of incubation. Hence, glucose was found to be the best source for cellulose production whereas sucrose, glucose, and mannitol were found to be suitable for optimum levels of cellulase production **[M.Ishihara., 2002., K. Toda, 1995., K. V.Ramana 2000].**

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Effect of nitrogen source

Production of cellulase has been shown to be sensitive to repression by different carbohydrate and nitrogen sources. The effect of nitrogen sources was studied in the growth medium, where

Peptone was replaced by ammonium sulphate, urea, and yeast extract. Among the various nitrogen sources tested, ammonium sulphate was found to be the best nitrogen source for cellulase production. Nitrogen is one of the major cell proteins and stimulation of cellulase activity by ammonium sulphate salt might be due to their direct entry in protein synthesis **[M.Mandels, 1975].**

Table 1 Morphological Character

S.NO.	SOURCE OF THE SAMPLE	NAME OF THE STRAIN	MORPHOLOGICAL CHARACTER
1	Orange peel	*Pseudomonas aeruginosa.*	Greenish blue, small opaque, Entire, rods, 0.8-1.00×0.5-2.5
2	Bamboo leaves	*Bacillus clausii.*	White opaque, entire, Rod 1.2-10×0.5-2.5
3	Pineapple peel	*Cellulomonassp,*	White opaque, entire, Rod 1.2-10×0.5-2.5
4	Banana peel	*Escherichia coli,*	White, smooth, translucent, entire, rod, 1-5×0.5-1
5	Beet root peel	*Lactobacillus sp*	Creamy, Butyrous consistency, Undula, rod, 2.5-10×0.5-1.2

Table 2 Biochemical Reactions

S.NO	NAME OF THE ORGANISM	BIOCHEMICAL REACTIONS							
		I	MR	VP	CITRATE	TSI	UREASE	OXIDASE	CATALASE
1	*Pseudomonas aeruginosa.*	-	-	-	+	+	+	-	-
2	*Bacillus clausii.*	-	-	+	-	_	+	-	+
3	*Cellulomonassp,*	+	-	-	-	-	-	-	-
4	*Escherichia coli*	-	-	-	-	-	-	-	+

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5	*Lactobacillus sp*	-	-		+	-	-	-	-	

Table 3 Molecular Characterization

Name of the organism	Primer	Name Sequence Details	Number of Base	Accession Number
Pseudomonas aeruginosa	27F	AGAGTTTGATCMTGGCTCAG	20	MF407324
	1492R	TACGGYTACCTTGTTACGACTT	22	
Bacillus clausii	8F	AGAGTTTGATCCTGGCTCAG	20	MF795055
	1541R	AAGGAGGTGATCCAGCCGCA	20	

Table 4 Estimation Of Reducing Sugar And Cellulase On Submerged

S. No.	Treatments	Reducing Sugar (g/l)			Cellulase (IU/ml/min)		
		24 Hrs	48 Hrs	72 Hrs	24 Hrs	48 Hrs	72 Hrs
1.	*Pseudomonas aeruginosa.*	2.08±0.09	3.10±0.11	5.12±0.13	2.76±0.08	3.64±0.05	5.34±0.07
2	*Bacillus clausii.*	1.21±0.05	1.23±0.07	1.25±0.09	0.57±0.02	0.57±0.04	0.57±0.02
3	*Cellulomonassp,*	1.69±0.05	1.72±0.07	1.75±0.09	0.44±0.07	0.74±0.05	0.74±0.06
4	*Escherichia coli*	1.19±0.01	1.21±0.03	1.23±0.05	0.32±0.03	0.64±0.07	0.66±0.04
5	*Lactobacillus sp*	0.97±0.07	0.99±0.09	0.11±0.01	0.527±0.02	0.59±0.04	0.61±0.03

Values are expressed in Mean ± Standard deviation; n=6

Table 5 Effect of pH

s.no	Strain name	pH
1	*Pseudomonas aeruginosa.*	9.1
2	*Bacillus clausii.*	9
3	*Cellulomonassp,*	10.3
4	*Escherichia coli*	10.5
5	*Lactobacillus sp*	11

Table 6 Effect of Incubation Temperature

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s.no	Strain name	Temperature (°C)
1	*Pseudomonas aeruginosa.*	40°C
2	*Bacillus clausii.*	38°C
3	*Cellulomonassp,*	36°C
4	*Escherichia coli*	46°C
5	*Lactobacillus sp*	31°C

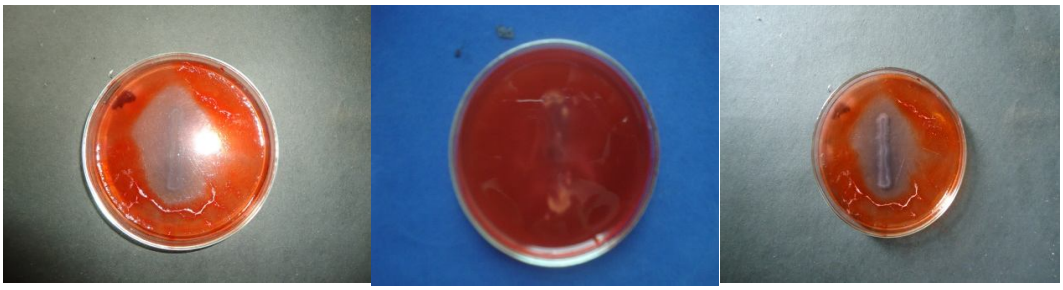
Table 7 Effect of carbon source on cellulytic enzyme

s.no	Strain name	Glucose (IU / ml/ min.)	Sucrose (IU/ ml/ min.)	Maltose (IU/ ml/ min.)	Fructose (IU/ ml/ min.)	Starch (IU/ ml/ min.)
1	*Pseudomonas aeruginosa.*	1.572	1.682	0.1952	0.4297	0.1591
2	*Bacillus clausii.*	1.488	1.998	0.3705	0.1233	0.1461
3	*Cellulomonassp,*	1.521	1.089	0.5271	0.1225	0.6241
4	*Escherichia coli*	1.543	1.1625	0.2001	0.1461	0.3528
5	*Lactobacillus sp*	0.1935	1.5403	0.4035	0.3330	0.1306

Table 8 Effect of Nitrogen source on cellulytic enzyme

S.no	Strain name	Ammonium sulphate (IU/ ml/ min.)	Urea (IU/ ml/ min.)	Yeast extracts (IU/ ml/ min.)
1	*Pseudomonas aeruginosa*	2.578	3.622	1.952
2	*Bacillus clausii.*	2.487	2.698	1.7050
3	*Cellulomonassp*	1.901	2.089	1.271
4	*Escherichia coli*	1.897	1.625	0.2089
5	*Lactobacillus sp*	0.986	1.403	0.4589

Figure 1 screening of celluase enzyme



Pseudomonas aeruginosa ***Bacillus clausii*** ***Cellulomonassp***

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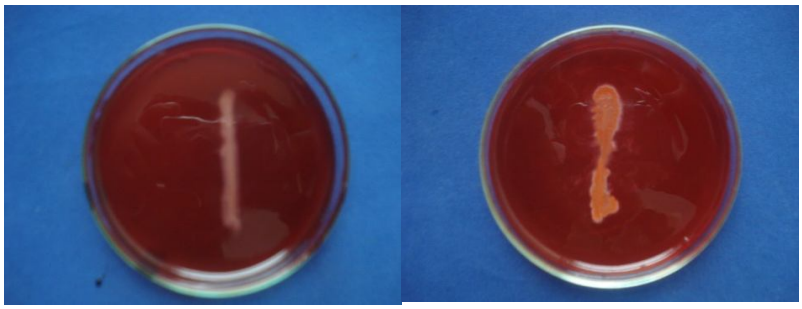


Escherichia coli *Lactobacillus sp*

Figure 2 Molecular characterization and phylogenetic tree

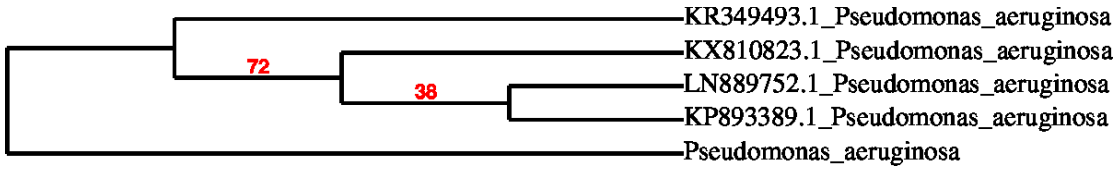
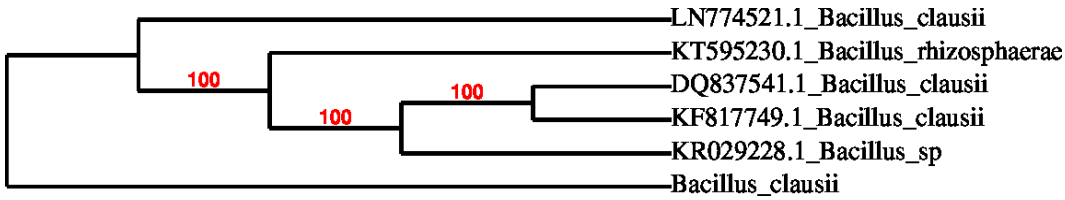


Figure 3



4. CONCLUSION

The present study was aimed at screening of microbial source with putative cellulase activity. Cellulase enzyme producing microorganism, there are five isolates were isolated from plant waste material. Amomg that, *pseudomonas aeroginosa* was successfully produced a cellulose enzymes in a higher level. The media was optimized for cellulase production and the isolate produces maximum cellulase at pH 9.1, temperature 40 ℃ and 72 hrs of incubation time. They were screened for their cellulase activity by Congo red test and they showed hydrolysis capacity value ranged from 2.76-5.34 IU/ml.The remaining four isolates exhibited lower cellulase activity ranged from 1.69-1.19 IU/ml. ((Sethi et al., 2013: Hmad et al., 2017) On the basis of their phenotypic characteristics and phylogenetic analyses, 2 isolates were closely

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related to *pseudomonas aeroginosa and bacillus clausii* in 16S rRNA gene sequence similarity (92.2%) with the closest type strain. Future study should be directed at the use of consortium in the degradation of organic wastes by the bacterial consortia for the ethanol production is highly significant.(B.K.Dabhi1*, R.V.Vyas 2014) It reduces the time span of degradation and produces no foul odour. The use of microbial consortium generated through natural selection or improvement of the performance of these microorganisms in plant and fruit peel waste degradation may be the best option for the efficient treatment of petrol crisis in the near future

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