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Microbial Cellulases: Isolation, Characterization and Application in Textile Industry

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ABSTRACT

Denim stone wash fabric is in high demand in youngsters. Stone washing is carried out by pumice stone to create such fabric. Major disadvantages of stone washing are that it reduces the life span of jeans as it weakens the fabric, it is overall expensive process as the garment is to be washed many times to remove grit. Alternative method is to use cellulases enzyme of microbial source to create denim look of jeans. Microorganisms including bacteria, fungi and actinomycetes can carry out bioconversion of cellulose by producing an enzyme cellulase. In the present study organisms were isolated from soil, animal waste and caterpillar on CMC medium. Total 15 isolates were obtained out of which three were quantitively and quantitively able to produce high cellulase enzymes. Cellulase enzyme was produced by submerged method, purification was done ammonium precipitation. Kinetic studies revealed 37 ^{0}C and pH 6 optimum for enzyme production. Finally, after application of enzyme on denim fabric showed light faint color and smooth texture of fabric thus use of enzyme can be better option for denim fabric creation than traditional methods of stone wash.

Key words: *Cellulase, Carboxymethylcellulose, Denim.*

Introduction

Denim finishing or also popularly known as Bio stoning, is one application of cellulase. Stone-washed effect is popular among teenagers and have created a new fashion demand. It gives garments a worn-in/ worn-out/ faded/ vintage appearance.

Stone washing is carried out by using large pumice stones. Frequent washing of garments leads to fading effect. Along with stone washing, other physical methods used are enzyme wash, bleach wash, acid wash (Mondal and Khan, 2014). Major disadvantages of stone washing is that it reduces the life span of jeans as it weakens the fabric, it is overall expensive process as the garment is to be washed many times to remove grit, moreover excess grit removal may be harmful to the environment. Popularity of garment washing especially of denim garment in the world market has been increasing day by day. To cope up with the ongoing demand of denim look, various methods are being developed by scientists (Islam, 2010).

In this work, the demanding denim look jeans were created via the action of the cellulase enzyme. Cellulase enzyme is known for its various applications, in different fields including paper and pulp, agriculture, bioconversion, detergents, fermentation and food. The third-largest class of enzymes utilized in the textile industry nowadays is cellulases. The global appeal of cellulases stems from their capacity to alter cellulose fibers found in clothing (Pazarlioglu, 2004). There are many advantages of

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using cellulase: they are easy and convenient to use, cellulases can work in ambient temperatures and pH levels, and do not need extravagant conditions, cellulases are biodegradable and environment friendly, using cellulases saves processing time. There are majorly three types of cellulases, endoglucases, cellobiohydrolases, B-glucosidases.

The present investigation focuses on extraction of cellulase from cellulose producing organisms. Although plants, fungi, and bacteria can all be used in the production of cellulases, bacteria have higher potential in production. Bacterial strains like, *Cellovibrio, Pseudomonas, Bacillus and Micrococcus* are all known to produce cellulase enzyme.

Materials and Method

Sample collection, Enrichment, Isolation and Screening of cellulase producing organism

Samples were collected from different sources like soil, animal waste and caterpillars. Enrichment was carried out in Carboxy-methylcellulose medium [CMC], broth incubated at 30 °C for 48 hrs. Isolation was done by serially diluting enriched broth and using spread plate on Congo-Red plates. Each isolate was straked on CMC agar plates and incubated at 37 °C for 72 hrs. After incubation, plates were flooded with Congo red reagent and observed for clear hollow zones around colonies (Yin, 2010; Shaikh *et al*., 2013).

Morphological and Biochemical characterization of potent isolates

Isolates that showed clear hollow zones around them are cellulase producing and hence selected for morphological identification by observing their colony characteristics, Gram nature and Biochemical tests like Carbohydrate fermentation test, Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, Urease test, Lysin decarboxylase test, Oxidase test, Catalase test, Casein hydrolysis test, and Starch hydrolysis test.

Quantitative estimation of enzyme activity

Isolates that are cellulase producing, were checked using Quantitative analysis method. Reaction mixture consisting of CMC, Citrate buffer and Culture filtrate (pH 5.0) was incubated at 50 °C for

30 min. Reaction was stopped using DNSA followed by water broth for 5 min. After cooling the reaction mixture, its Absorbance was read at 540 nm using visible spectrophotometer (Plackett *et al.,* 1946; Bairagi *et al.,* 2002).

Identification of Potent isolate

Genomic identification of isolate showing highest enzyme activity was done using 16S RNA sequencing (Lokhande *et al.,* 2016).

Production and Purification of enzyme

Potent isolate was used to produce enzyme. Production was carried out in salts medium containing Glucose or CMC as carbon source. Broth was incubated at 37 °C for 24 hrs. Cell density of broth was measured at time interval of 60 min up to 300 min to check log phase growth. Purification of enzyme was carried out by Ammonium precipitation in Buffer followed by Dialysis (Lee *et al.,* 2008).

Determination of Kinetic study

Kinetic study of isolate was performed by varying different parameters like pH [5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4], Temperature [4, RT, 37 °C, 55 °C, 70 °C, 90 °C], Carbon source [Sucrose, Fructose, Glucose, Galactose, Arabitol] and Nitrogen sources [Ammonium Sulphate, Peptone, Yeast extract, Corn steep liquor] (Shankar *et al.,* 2011; Biol *et al.,* 2005).

Application of enzyme

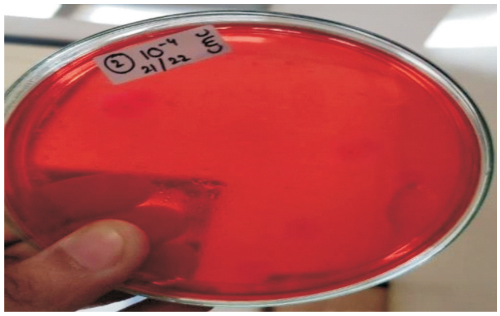
5 cm by 5 cm swatches of jeans were obtained. One swatch was 'test' swatch, second was labelled with 'control' and third 'untreated'. The three swatches were taken in clean petri plates. The 'test' swatch was soaked in 5 ml of purified cellulase enzyme obtained from isolate S3. The 'control' swatch was soaked in 5ml Buffer A solution. Third swatch was unwashed. All the three swatches were kept in petriplates for soaking at room temperature for 5-7 days. After 7 days, the 'test' and 'control' swatches were hand washed for 10 min in warm water until clear water is left. After washing the swatches were dried and compared for texture and color (Sweeney *et al.,* 2002).

Results and Discussion

Sample collection, Enrichment, Isolation and Screening of Cellulase producing organism

Three different samples (soil, animal waste, caterpil-

lar) were taken. Enrichment was carried on Carboxy-methylcellulose medium (CMC), incubation time was 48 hours at 37 °C. Isolation of organism was done on Congo Red agar plates. Obtained isolates were streaked onto Carboxy-methylcellulose agar plates (CMC). After incubation, CMC agar plates were flooded with Congo red and allowed to stand for 15 min. Observation of clear hollo zones were screened as cellulase producing organism. Out of ten isolates, three isolates (S2, S3, S5) were cellulose producing and chosen for further objectives.



Pic. 1. Before staining with congo-redplates.

Pic. 2. After staining with congo-redplates.

Morphological and Biochemical Characteristic Identification

Isolates that showed clear hollow zones around themselves were considered as cellulase producing. They were carefully labelled and subculture on Carboxymethylcellulose agar plates. The sub cultured colonies were picked to perform morphological characterization (size, shape, color, margin, elevation, consistency, opacity, gram nature) and Biochemical characterization (catalase test, oxidase test, indole test, methyl red test, VP test, citrate test, sugar utilization test).

Table 2. Biochemical characteristics of cellulase producing isolates

• Biochemical tests	• S2	• S3	• S5
• Catalase test	• -	• +	• +
• Oxidase test	• +	• +	• -
• Indole test	• -	• -	• -
• Methyl red test	• +	• +	• +
• Vogus Prausker test	• -	• -	• -
• Citrate test	• +	• +	• +
• Nitrate reduction test	• +	• +	• -
• Casein hydrolase test	• +	• +	• +
• Urease test	• -	• -	• -
• L.D test	• -	• -	• -
• Starch hydrolysis test	• -	• -	• -
• Glucose test	• -	• -	• -
• Sucrose test	• -	• -	• -

Quantitative estimation of Enzyme activity

The estimation of amount of cellulase production was carried out in reaction mixture containing Carboxymethyl cellulose, Citrate Buffer and Isolate cul-

Table 1. Morphological characteristics of cellulase producing isolates

• Morphological Characters	• Isolate S2	• Isolate S3	• Isolate S5
• Size	• Medium	• Medium	• Medium
• Shape	• Irregular	• Irregular	• Irregular
• Colour	• Off-white	• Cream	• Off-white
• Margin	• Even	• Even	• Even
• Surface	• Smooth	• Smooth	• Smooth
• Elevation	• Convex	• Convex	• Convex
• Consistency	• Sticky	• Sticky	• Sticky
• Opacity	• Translucent	• Translucent	• Translucent
• Gram nature	• Gram positive rod	• Gram negative cocci	• Gram positive rod

ture. Reaction was stopped using adding DNSA. Absorbance was detected at 540 nm using visible spectrophotometer. The resultant O.D was found to be greater for isolate S3, it was taken for further production and extraction of enzyme.

Table 3. Quantitative estimation of cellulase producing isolates

Isolates	O.D. at 540 nm
S2	0.09
S3	0.11
S5	0.10

16s RNA genome sequencing of isolate S3

The 16s RNA sequencing of isolate S3 was carried out. The resultant sequence is as follows:

CCATAGTTTGATCCTGGCTCAGATTGAACGC
TGGCGGCAGGCCTAACACATGCAAGTCGAGC
GGATGAGTG
GAGCTTGCTCCATGATTCAGCGGCGGACGG
GT GAGTAATGCCTAGGAATCTGCCTGGTAGT
GGGGGACAAC
GTTTCGAAAGGAACGCTAATACCGCATACGT
C CTACGGGA GAAAGTGGGGGATCTTCGGA
CCTCACGCTAT
CAGATGAGCCTAGGTCGGATTAGCTAGTTGG
TGAGGTAAAGGCTCACCAAGGCGACGA
TCCGTA ACTGGTC
TGAGAGGATGATCAGTCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCA
TGCCGCGTGTGTGAAGAAGGTCTTCGGAT
TGTAAAGCACT
TTAAGTTGGGAGGAAGGGCAGTAAGTTAATA
CCTTGCTGTTTTGACGTTACCAACAGAATAA
GCACCGGCT
AACTTCGTGCCAGCAGCCGCGGTAATACGAA
GGGTGCAAGCGTTAATCGGAATTACTGGG
CGTAAAGCGCG
CGTAGGTGGTTCGTTAAGTTGGATGTGAA
AGCCCCGGGC

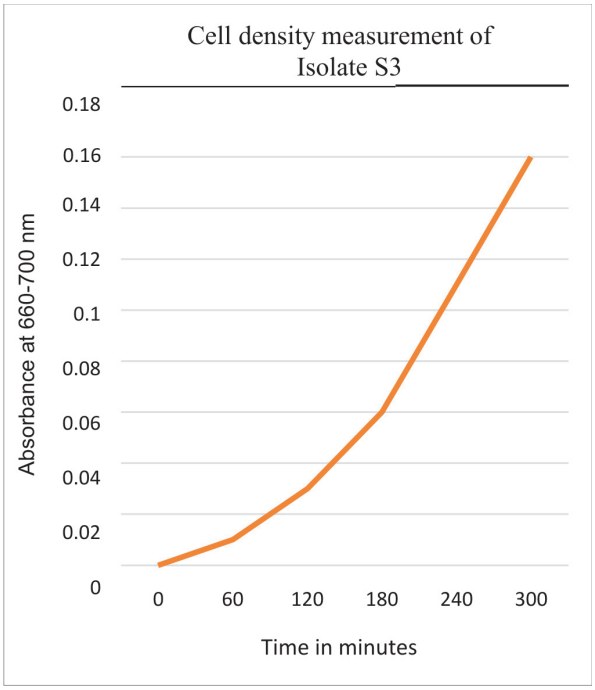
TCAACCTGGGAACTGCATCCAAAACTGGC
GAG
CTAGA GTATG GCAGAGGGT GGTG GAATTTC
CTGTGTAGCGGTGAAATGCGTAGATATAG
GAAGGAACACCA

Absorbance at 660-700 nm

GTGGCGAAGGCGACCACCTGGGCTAATACT
GACACTGAGGTGCGAAAGCGTGGGGAGCAA
ACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATG
TCGACTAGCCGTTGGGATCCTTGAGATCTTA
GTGGCGCAGCT
AACGCATTAAGTCGACCGCCTGGGGAGTA
CGGCCGCAAGGTTAAAACTCAAATGAATTG
ACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCAGGCC
TTGACATGCAGAGAACTTTTCCAGAGATGG
ATTG GTGCC TTCG GG AACTCTGACA CAGGT
GCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGTAAC
GAGCGCAACCCTTGTCCTTAGTTACCAG
CACGTTAAGGTGGGC

Graph 1: Cell density determination of isolate S3 during production of enzyme

ACTCTAAGGAGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAGTCATCAT
GGCCCTTACGG
CCTGGGCTACACACGTGCTACAATGGTCGG
TACAAAGGGTTGCCAAGCCGCGAGGTGGAG
CTAATCCCATA
AAACCGATCGTAGTCCGGATCGCAGTCTG CA
ACTCGACTGCGTGAAGTCGGAATCGCTAG
TAATCGTGAAT



Graph 1. Cell density determination of isolate S3 during production of enzyme

Pic. 3. Dialysis of purified enzyme.

CAGAATGTCACGGTGAATACGTTCCCGGGCC
TTGCACACACCGCCCGTCA

Production of Enzyme from isolate S3

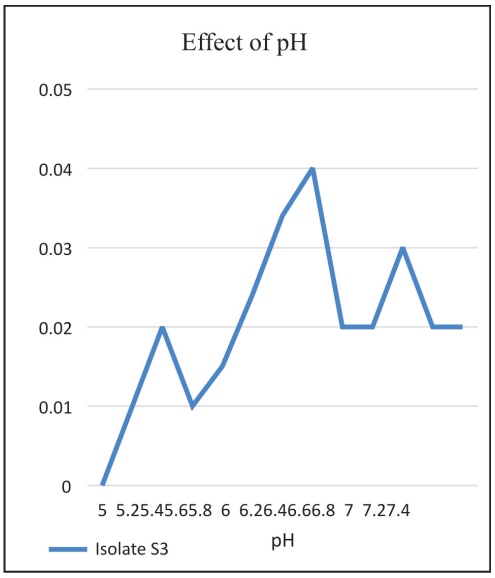
Production of Isolate S3 (having highest cellulase producing activity) was done by submerged fermentation in Salt medium. Culture broth was sampled at different time intervals during growth to determine cell density at 660-700 nm. Initial cell density at 0 min was 0.00. After every interval of 60 min, cell density was measured at 660-700 nm. At 300 min, the cell density of broth was 0.16. Hence, it can be confirmed that the culture was in log phase and will yield greater enzyme activity.

Purification of Enzyme

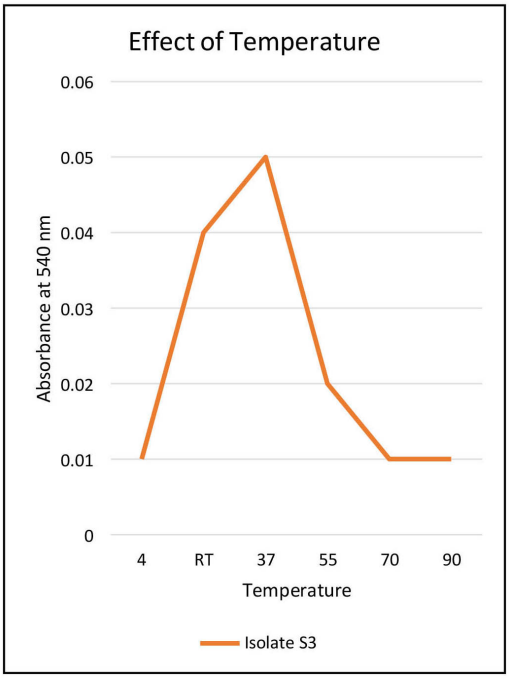
Enzyme purification was done in a buffer A (50 mM Tris-HCL, pH 8.0) and Buffer B (80% ammonium sulphate)and kept at 4 °C for 12 hours. After collecting the precipitate, it was centrifuged for 30 minutes at 10,000 rpm. The gathered supernatant was dialyzed against Buffer A for the entire night. After another centrifugation for ten minutes at 10,000 rpm, the supernatant was filtered, sterilized, and kept at 0 °C.

Kinetic Study of enzyme

Kinetic Study was performed at 40 °C in 50 mM citrate buffer (pH 4.8 and 0.01% sodium azide) and inoculum of S3, incubated for 47 hours at room temperature. Effect of pH, Temperature, Carbon sources, Nitrogen sources. Cellulase's maximum enzyme activity was seen at 37 °C, pH 6.4, sucrose as a carbon source, and peptone as a nitrogen source.



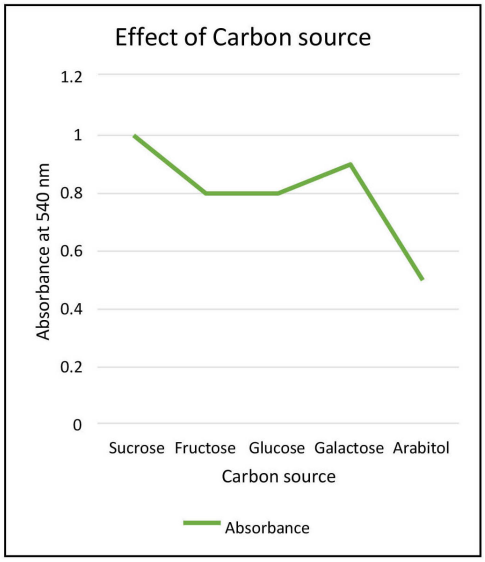
Graph 2. Effect of pH on the enzyme activity



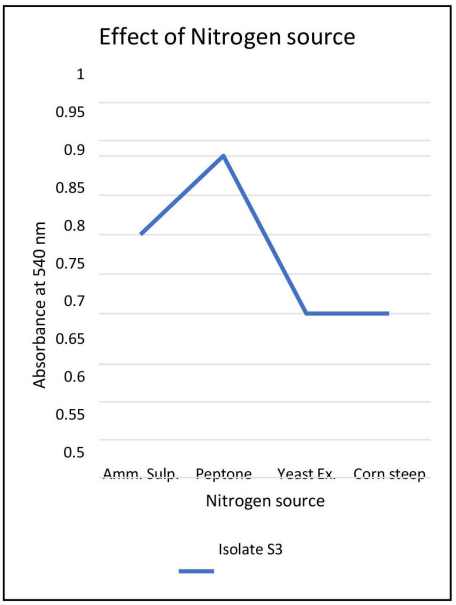
Graph 3. Effect of Temperature on the enzyme activity

Application

Purified cellulase enzyme obtained from Isolate S3 (*Pseudomonas sp.*) was used to test against jeans swatches to demonstrate the effect of vintage, fade out jeans. Three different swatches, one of 'test', one 'control' and one 'unwashed' were prepared. 'Test'



Graph 4. Effect of Carbon Source on the enzyme activity

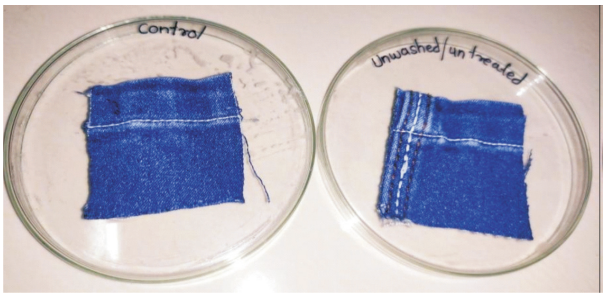


Graph 5. Effect of Nitrogen Source on the enzyme activity

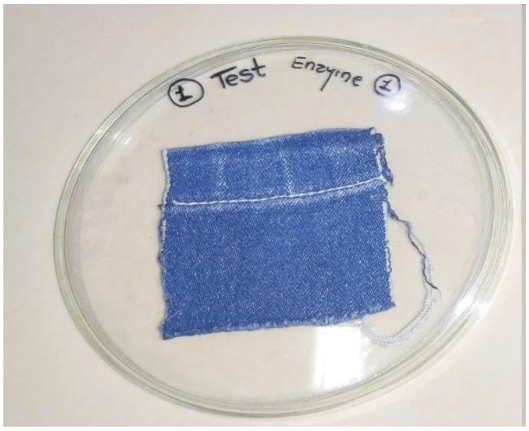
was soaked in enzyme, 'Control' was soaked in buffer. After 5-7 days, washing and drying results were compared. The 'Test' swatch was seen to fade in color than the 'Control' and 'unwashed' swatches. The fabric of 'Test' was smooth to touch, no hardness was observed.

Conclusion

An enzyme called cellulase is known to break down



Pic. 4. Control and Unwashed swatches of jeans



Picture 5. Test swatches of jeans

cellulose. Cellulose is considered as the ubiquitously abundant organic compound found in plants and algae. Cellulose is used in dietary fiber, paper production, food additive, preservatives. Microbial cellulases are used in food, animal feed, brewery, textiles, wine, laundry. The ever- increasing demand for non-expensive cellulases and their enhanced utilization in multiple sectors is the source of study.

In the present study, denim look is being given to jeans in affordable way. Today, denim is way more common though it is expensive to produce, it is loved by teenagers. To create denim, look, jeans are stone washed. Stone washing is expensive as well as the results of it are not promised and non- controllable. To solve this problem, cellulase enzyme extracted from *Pseudomonas sp.* is used to create the desired denim look. Three cellulase producing isolates were obtained. They were tested for quantitative analysis and isolate S3 showed maximum cellulase enzyme activity. 16S RNA genomic sequencing was carried out and it was found that isolate S3 was similar to *Pseudomonas sp.* Production of enzyme was carried out in salts medium by submerged fermentation followed by purification by ammonium

precipitation at 80% and dialysis. The enzyme kinetic study showed that the isolate produced maximum enzyme at pH 6.6 and temperature at 37 °C.

The purified enzyme was used to study the application. Jean's swatches were soaked for 5-7 days in the purified enzyme [test], Buffer [control] and unwashed swatch was kept for calibration. After washing and drying of the swatches, it was found that the 'test' swatch showed denim like appearance unlike 'control' and 'unwashed' swatches. It can be observed that the enzyme is effective in getting desired denim look while not affecting to clothes texture. The production of cellulase enzyme is economical in a way that cellulose is abundant in nature as it is present in plants, trees, and even in algae, making it efficient to large scale production.

Conflict of Interest: None

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