Neutral Avicelase from *Serratia marcescens* with Denim Biofinishing Potential

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Screening of 13 avicelase positive bacterial isolates obtained from soil and leaf litter proved isolate L4 to be a potent avicelase producer with highest zone of clearance (25 mm) and Enzymatic index of 1.8. The phylogenetic tree constructed on the basis of 16S rRNA gene sequences revealed its closeness to *Serratia marcescens* WW4 and was designated as *Serratia marcescens* L4. Maximum avicelase (8.2 IU) production by *S. marcescens* was supported at pH 7.0, 30°C with CMC (5 g/L). Avicelase production was repressed by sugars and showed non-dependence on metal ions. Among agrowastes, potato peel supported the highest activity (6.4 IU). The enzyme showed pH and temperature optima of 7.0 and 55°C and retained almost 72% activity at 55°C up to 2 h. Enzymatic biofinishing at pH 7.0, 55°C and 2 h incubation revealed some amount of weight loss, indigo dye and reducing sugar release from the denim fabric. Thus the neutral avicelase of *S. marcescens* may find application in denim biofinishing with reduced backstaining.

Keywords: *Serratia marcescens*, Neutral Avicelase, Optimization, Agrowastes, Denim Biofinishing

Introduction

Cellulases are widely spread in nature, predominantly produced by microorganisms like molds, fungi and bacteria1. There has been increasing interest in cellulase production by bacteria because of fast growth rate. Although a large number of bacteria can degrade cellulose, only a few of them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose2. Therefore, research is aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency. The potential of cellulases has been revealed in various industrial processes, as cotton softening and denim finishing in the textile industry, de-inking, drainage improvement and fiber modification in the pulp and paper industries, as well as in laundry detergents as softening, anti-pilling and colour-reviving agents.3 Use of cellulases for denim washing is a standard eco-friendly technique to achieve desirable appearance and softness for cotton fabrics and denims4. But enzymatic washing of denim till date involve acid cellulase and neutral cellulase. However, the use of acidic cellulase has a drawback of backstaining of the indigo dye on to the fabric. Backstaining could be significantly reduced at neutral pH, hence neutral cellulases are being screened in order to minimize backstaining5. The present study attempts to isolate a potent neutral avicelase producer from natural habitat of leaf litter with denim biofinishing activity.

Materials and methods

Sampling and isolation

Soil and leaf litter samples were collected from in an around Bangalore, selected for heat resistance spores by keeping in water bath at 80°C for 20 minutes, serially diluted and plated on nutrient agar medium and incubated at 37°C for 24 h. The isolates were initially screened based on their ability to grow on a synthetic medium (pH 7.0) containing Avicel (Sigma Aldrich, USA) as the sole carbon source.

Screening step using the congo red test

The isolates obtained during initial screening with Avicel were selected by the Congo red test6. Isolates showing an enzymatic index (EI) higher than 1.50 were considered to be potential producers of cellulases. The isolates found to be avicelase positive were then grown on nutrient agar with starch (1% w/v) to check whether they were amylase negative. The isolates found to be avicelase positive and amylase negative were selected for further studies.

Enzyme production and assay

The quantification of hydrolysis of CMC or Avicel (0.5% w/v), by the crude concentrated supernatant proteins was determined using a standard method in phosphate buffer (50 mM, pH 7) at 55°C. One international unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugars in glucose equivalents per minute under the above assay conditions6.
Molecular identification of bacterial isolate

Pure culture of the isolate was grown overnight on nutrient broth for the isolation of DNA. The DNA was isolated from the bacteria using cell lysis method and the 16S rDNA was amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GTTACCTTGTTCAGACCTT-3′) (Chromous Biotech Pvt. Ltd. Bengaluru). The amplified 16S rDNA PCR product was sequenced using automated sequencer (Applied Biosystem). The Sequence Similarity Search was done for the 16S rDNA sequence using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/) and the unknown organism was identified.

Factors affecting avicelase production

Isolate L4 showing highest avicelase activity was selected for optimization of avicelase production. Five factors – different concentrations of CMC (2, 5, 10, 15 and 20 g/L), pH (5.0, 6.0, 7.0, 8.0, 9.0), carbon sources (glucose, sucrose, maltose, fructose and arabinose), metal ions (CuSO₄, CaCl₂, MnSO₄, FeSO₄, ZnSO₄) in nutrient medium containing 10 g/L CMC and incubated for 48 h were evaluated for the study.

Determination of pH and temperature optima of avicelase

The optimum pH and temperature of crude avicelase from S. marcescens was estimated in the pH range (5.0-9.0) and temperature range of 40-100°C using standard procedures. pH and temperature stability of the avicelase was tested at the optimum pH and temperature respectively, using standard procedures and residual activity was measured.

Evaluation of different agrowastes for avicelase production

CMC in the media was substituted with fourteen different agrowastes (10 g/L)– Jatropa oil cake, Mahua oil cake, rice bran, groundnut shell powder, pongamia oil cake, banana peel, coconut oil cake, sugarcane bagasse, pineapple peel, potato peel, jowar, groundnut oil cake, ragi bran, coconut cob procured from the local market of Bangalore and checked for avicelase production.

Biofinishing activity

Indigo dyed denim fabric (1cm×1cm) was used to determine the biofinishing activity of the enzyme. The denim weights were determined before and after the enzymatic treatment. The assay was carried out as mentioned earlier except for replacing the substrate with denim fabric. The amount of indigo dye released into the solution was determined by assaying the absorbance at 619 nm. The denim was removed from the solution and the reducing sugars were measured by DNS reagent by reading the absorbance at 540 nm.

Statistical analysis

All experiments were conducted in triplicates, and the results were expressed as ± SD; p<0.05.

Results and Discussion

Isolation and screening of avicelase producers

Out of 14 bacterial isolates, L4 was positive for avicelase production (25 mm) with EI of 1.8 and negative for amylase production and was selected for further optimization studies.

Identification of isolate L4

The constructed 16S-rRNA phylogenetic tree indicated that the bacterium had 99% similarity to Serratia marcescens WW4 and designated as Serratia marcescens L4 (Gen Bank accession No. KR905568).

Effect of pH on avicelase production

Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. The optimum pH for maximum enzyme production was 7.0 (6.4 IU) followed by pH 6. The enzyme activity gradually increased on increasing the pH up to the optimum followed by a gradual fall in activity (Figure 1). Backstaining could be significantly reduced at neutral pH, hence neutral cellulases started to be screened in order to minimize backstaining. In this regard, S. marcescens L4, with neutral cellulase/s, will be advantageous to minimize backstaining during biofinishing of denims.

Endoglucanase from Cellulomonas, Bacillus and Micrococcus sp., isolated from the estuarine coir netting effluents hydrolyzed substrate in the pH range of 4.0 to 9.0, with maximum activity at pH 7.0.

![Fig. 1 — Effect of medium pH on avicelase production by S. marcescens L4. Values represent ± SD (n=3); p< 0.05](image-url)
Effect of CMC concentration
The effect of various concentrations of substrate (2-20 g/L) on avicelase activity is shown in Figure 2. The maximum cellulase activity (8.2 IU) was found at 5 g/L concentration of CMC at temperature of 30°C and pH of 7 ± 0.2. The production of cellulolytic enzymes is induced only in presence of the substrate and is repressed when easily utilizable sugars are available. The present observations corroborate with this theory.

Effect of different carbon supplements
Supplementation of different carbon sources resulted in complete repression of avicelase activity in the presence of easily utilizable sugars. Maximum cellulase activity (6.4 IU) by L4 was obtained with CMC (10 g/L) alone. Similarly, highest cellulase activity (0.26 U/mL) of a Bacillus sp. was observed when the culture was grown in LB medium supplemented with 1% CMC. In contrast, induction of avicelase enzyme was reported in presence of lactose as the carbon source when produced by Cellulomonas fimi and Cellulomonas cellusea strains.

Effect of different metal salts
Avicelase activity of S.marcescens L4 was not influenced by addition of mineral salts in the medium thereby showing non-dependence of the enzyme on metals. The inorganic salts generally play relatively subordinate role.

Time course study
Time course study of avicelase production by S.marcescens L4 under optimized conditions [pH 7.0, CMC (5g/L)] showed a time dependent manner of avicelase production with highest activity (15.4 IU) on Day 2. Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compared to maximum. A 10-d incubation period was essential for cellulase production in Bacillus sp. S.marcescens SGS 1609 isolate showed maximum cellulase production by 4th day. S.marcescens L4 with appreciable avicelase activity by 48 h could be explored for its biofinishing activity.

Evaluation of different agrowastes for avicelase production
Of the fourteen different agro and food wastes explored for avicelase production by L4, all other substrates except jatropha oil cake, pongamia oil cake and ground nut shell, supported varied levels of avicelase production by 48 h. Potato peel supported highest avicelase activity (6.4 IU) (Figure 3). The maximum cellulase activity (12.08 U/g) was found at 5% concentration of saw dust at temperature of 30°C and pH of 7.2 ± 0.2. Bacillus subtilis KO exhibited maximum activity at 10% concentration of molasses. Carbon sources in majority of commercial cellulase fermentations are cellulosic biomass including straw, spent hulls of cereals and pulses, rice or wheat bran, bagasse, paper industry waste and various other lignocellulosic wastes.

pH and temperature optima of avicelase
S.marcescens L4 avicelase showed maximum activity between pH 6.0-7.0 and the values decreased after pH 7.0. The avicelase from Geobacillus stearothermophilus showed an optimum pH 7.0 and presented good pH stability between pH 5.0-8.0. Most microbial cellulases have pH optima in acidic or neutral range. The avicelase of L4 was stable at 55°C upto 2 h and retained almost 74% activity.

Fig. 2 — Effect of CMC concentration (g/L) on avicelase production by S. marcescens L4. Values represent ± SD (n=3); \( p<0.05 \)

Fig. 3 — Evaluation of different agrowastes (10 g/L) for avicelase production by S. marcescens L4. Values represent ± SD (n=3); \( p<0.05 \)
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References

