International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 09-07-2021, Accepted: 24-07-2021, Published: 09-08-2021
Volume 6, Issue 4, 2021, Page No. 136-139

Isolation, screening, purification of cellulase enzyme from bacteria present in the gut of *Leiochrinus Nilgirianus*

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Abstract
This study was conducted to isolate, screening, purification of cellulase from bacteria present in the gut of *Leiochrinus nilgirianus*. Based on experiments, three bacterial strains produced clear transparent zone into carboxymethyl cellulose (CMC) agar plate were identified as cellulase producing bacteria. Different culture parameters such as pH, temperature, incubation period, substrate concentration and carbon sources were optimized for enzyme production. According to the morphological and biochemical tests, the isolated strains were identified as *Porphyromonas*, *Bifidobacterium* and *Lactobacillus sps.* The second strain *Bifidobacterium sp.* showed high potentiality for maximum cellulase production (0.29 U/ml) at pH 7.0 after 24 h of incubation at 40°C in a medium containing 1.0% CMC. In this study revealed the cellulase activity from bacteria present in the gut of *Leiochrinus nilgirianus* and find out the scavenging action of insect. This could helpful for agricultural development.

Keywords: cellulase, bacteria, *Bifidobacterium sp*, screening, characterization

Introduction
In the agricultural sector, the coconut palm is the most vital part of Indian agriculture due to their long life cycle, high yield, nutritional richness, economic viability and also provide the employment opportunity to rural non-farmer and farmer sector. A Leiochrini beetle, *Leiochrinus nilgirianus* Kaszab 1946 and immature stages were found feeding on sooty mould developed over the honey dew excreted during early morning hours, founf in coconut palm leaves. *In situ* habitat conservation of *Leiochrinus nilgirianus* would help to reduce the sooty mould at no cost in the most natural and eco-friendly manner avoiding chemical management options and other expensive methods. Cellulases are a complex group of enzymes which are secreted by a broad range of microorganisms including fungi, bacteria, and actinomycetes. In the natural environment, synergistic interactions among cellulolytic microorganisms play an important role in the hydrolysis of lignocellulosic polymer materials (Sandhya *et al*., 2018) [6]. In fact, it is the combined action of three major enzymes which determines the efficiency of this process. They are exoglucanases, endoglucanases, and β-glucosidase. Microorganisms produce these enzymes in a diverse nature which determines their efficiency in cellulose hydrolysis. During the cellulose degradation reaction, the enzyme targets the β-1, 4-linkages in its polymeric structure. This is an essential ecological process as it recycles cellulose in the biosphere (Sonia *et al.*, 2012).
The isolation and identification of cellulases has been limited in the past to culturable microorganisms. However, recent advances in molecular techniques, such as the creation of metagenomic libraries will widen the pool of cellulolytic enzymes available for biofuel research. This approach will allow exploition of cellulases and related enzymes from otherwise unculturable microorganisms which may produce enzymes with novel characteristics. In this paper, aimed to isolate newer source of extracellular catalase enzymes from the bacteria present in the gut of *Leiochrinus nilgirianus* to potential application of the cellulases for fungal degradation in coconut palm tree.

Materials and Methods
Sample collection and preparation of crude extract
Samples were collected from CPCRI, Kayamkulam and species was identified as *Leiochrinus nilgirianus***.** The whole organism was grind with Tris - HCl buffer (pH 7.4, 0.1 M) using a pestle and mortar. The sample was centrifuged for 10 min at 10000 *g* at 4°C. The resultant supernatant was separated and stored in 2.0 ml vials at -20°C.

Isolation of cellulase producing bacteria
Cellulase-producing bacteria were isolated from soils by the dilution pour plate or spread plate method using CMC agar media. The plates were incubated at 45, 50, and 55°C for 24 hours. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl (Apun *et al*., 2000) [1]. To indicate the cellulose activity of the organisms, diameter of the clear zone around colonies on CMC agar was measured. Besides, a more quantitative assay method was used to determine the cellulose activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a DNS method (Miller *et al*., 1989) [2]. A bacterial isolate with the highest activity was selected for optimization of cellulose production.

Identification of bacteria

The identification of bacteria was carried out by morphological studies i.e. staining including Gram staining, motility test, acid fast test, endospore staining. Cultural characterization on agar plates like colony morphology that is shape, size, margin, elevation, opacity, texture and pigmentation and also growth in broth and biochemical test includes catalase test, oxidase test, carbohydrate fermentation test, indole, methyl red, citrate utilization test, Voges Proskauer test, H_2S production test, Starch hydrolysis test, urease production test, nitrate reduction test (Aneja *et al*., 2005).

Enzyme production medium

Production medium contained (g/L) glucose 0.5 gm, peptone 0.75 gm, $FeSO_4$ 0.01 gm, KH_2PO_4 0.5 gm, and $MgSO_4$ 0.5 gm. Ten millilitres of medium were taken in a 100 mL conical flask. The flasks were sterilized in autoclave at 121°C for 15 min, and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37°C in shaker incubator for 24 h. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Enzyme assay

Cellulase activity was measured following the method of Miller, 1989 [2].

Briefly, a reaction mixture composed of 0.2 mL of crude enzyme solution plus 1.8 mL of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7) was incubated at 37°C in a shaking water bath for 30 min. The reaction was terminated by adding 3 mL of DNS reagent. The colour was then developed by boiling the mixture for 5 min. OD of samples was measured at 575 nm against a blank containing all the reagents minus the crude enzyme.

Process optimization for maximum cellulase production

Effect of pH on enzyme activity

Flasks with broth containing the optimum concentration of substrate and carbon source are taken and the pH of the broth is adjusted to 5.0 to 10.0 in different flasks using 1 N HCl and 1 N NaOH and sterilized. The cultures are inoculated and incubated at particular temperature. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source.

Effect of temperature on enzyme activity

Production medium at pH 7 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from, 20 to 100°C for 24 h. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source.

Results

Isolation of cellulase producing bacteria from *Leiochrinus nilgirianus*

Cellulase producing bacteria were isolated from *Leiochrinus nilgirianus* and cultured between 1-7 days on different growth media. Each isolate was assigned a code depending on the culture media. Total three catalase producing bacteria (LN1 to LN3) were isolated from *Leiochrinus nilgirianus* and sub-cultured to obtain pure cultures.

Screening of cellulase activity

Cellulase activity was observed from the zone of hydrolysis observed on agar surface mentioned in Table 1. The LN2-*Bifidobacterium sp* showed high potentiality for maximum cellulase activity (19 mm) at pH 7.0 after 24 h of incubation at 40°C. For further study the *Bifidobacterium sp* was considered and maintained by repeated sub culturing.

Table 1: Showing zone of inhibition (mm)

Sl. No	Isolates	Zone of Inhibition(mm)
1	LN1	19
2	LN2	12
3	LN3	10

Identification of bacteria

According to the morphological (Table 2, 3) and biochemical tests (Table 4), the isolated strains were identified as *Porphyromonas sp*, *Bifidobacterium sp*, *Lactobacillus sps*. The LN2 from *L. nilgirianus* were showed the clear zone formation by producing amylase which indicates the positive result (Figure 2).

Table 2: Colony characters on nutrient agar plate

Size	Shape	Edge	Elevation	Opacity	Texture	Pigmentation
0.5-1.3 micrometer	coryneform rods	bifurcated	clubbed	opaque	rough	white, convex, shiny colony

Table 3: Result of Staining of LN2

Sl. No	Staining	Result
1	Gram Staining	Positive
2	Endospore Staining	Negative
3	Motility	Non-Motile

Table 4: Biochemical characterizations: various biochemical tests

Sl. No	Biochemical Test	Result
1	Indole Production Test	Negative
2	Methyl red Test	Negative
3	Voges Proskauer Test	Positive
4	Citrate utilization Test	Positive
5	H_2S Production Test	Positive
6	Urease Test	Positive
7	Catalase Test	Positive
8	Oxidase Test	Positive

9	Starch Hydrolysis Test	Negative
10	Nitrate reduction Test	Positive
11	Gas Production from glucose	Negative

Enzyme assay

Cellulase activity was measured at 520 nm and the aborbance were recorded. 1.2ml of enzyme showed maximum activity at 520nm.

Effect of pH on enzyme activity

To study the effect of pH culture media pH was adjusted using different pH buffer ranging from 5.8 -8.0. It was incubated at 37ºC for 48 hrs. The maximum activity of enzyme was found at pH 7 (0.08 u/ml).

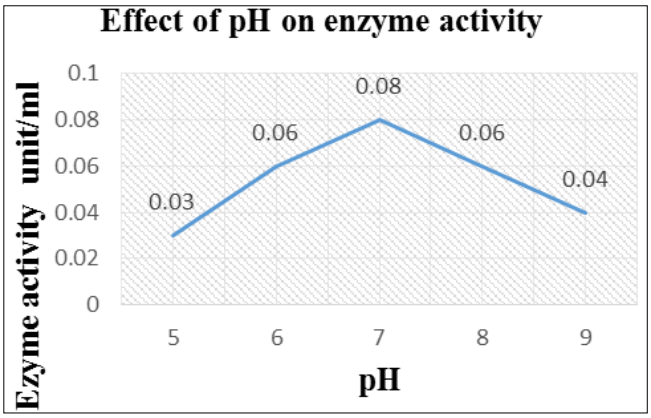

Fig 1: Effect of pH on cellulase activity

Effect of temperature on enzyme activity

The cellulase activity is relatively stable in the temperature range 60-70ºC and retains 85.2% of its activity at 60ºC. In present study the maximum activity was found at 60ºC (0.08 u/ml).

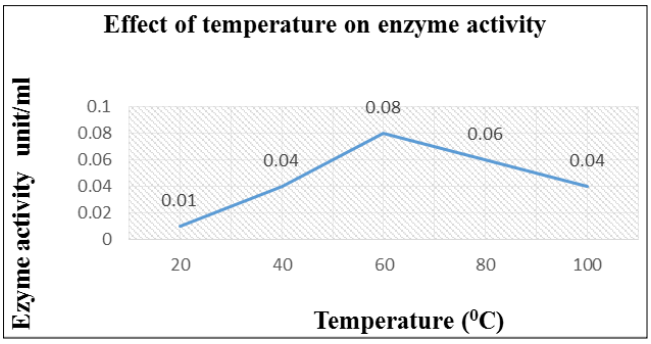

Fig 2: Effect of temperature on cellulase activity

Discussion

In the present study three bacterial isolates (*Porphyromonas*, *Bifidobacterium* and *Lactobacillus sps*) shown cellulase activity. Out of this, bacterial colony showing maximum cellulase activity was studied further. The most active protease producer was identified as *Bifidobacterium sp*. various physiological factors were studied like effect of pH and temperature. It is Gram positive, non-motile, catalase positive, non- spore former, indole negative, VP positive, methyl red negative. Cellulase activity was measured at 520 nm and the aborbance were recorded. 1.2ml of enzyme showed maximum activity at 520nm.

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Increase in pH shows increase in enzyme activity. In current study, the maximum activity of cellulase enzyme in *Leiochrinus nilgirianus*, was found at pH 7 (0.08 unit/ml). Sonia *et al*., (2012), reported that the maximum enzyme activity was observed in medium of pH 7.0–11.0. There have been diverse reports on the optimal initial pH and temperature for cellulolytic enzyme production by *Paenibacillus* sp. In a previous study, *P. curdlanolyticus* B-6 was cultivated for enzyme production at pH 7.0 (Waeonukul *et al*., 2009) [3].

In general, the optimal growth temperature ranges between 36–38°C and providing a key role of catalase enzyme in the *Bifidobacterium sps*. In present study the maximum activity of enzyme in *Leiochrinus nilgirianus*, was found at 60ºC (0.08 unit/ml). Enzyme activity was stable with temperature within range of range of 40ºC to 70ºC. Enzymes was also still active at 80ºC. Kumar *et al*., (2012) [4], reported that the optimal temperature for cellulase production by *P. polymyxa* was 37°C. Yoon *et al*., (2003), accounted that the optimal growth temperature for *P. terrae* was 30°C, which is similar to that observed for optimal CM Case production by the strain ME27-1.

Conclusion

In this present study *Leiochrinus nilgirianus* shows presence of catalase producers. The most effective catalase bacteria were screened and identified as *Bifidobacterium sps*. Various physiological factors were studied like pH and temperature. Keeping in view about use of catalase enzyme it can be harnessed for biotechnological processes. The scavenging action of *Leiochrinus nilgirianus* on sooty mould deposition on coconut palm leaves is due to the presence of catalase from *Bifidobacterium sps.* The present information of catalase activity from bacteria present in the gut of *Leiochrinus nilgirianus* could assist the future attempts to unravel the scavenging action of insect and will provide the much needed impetus to agricultural development.

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