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Optimisation of culture conditions for cellulase production by *Arthrobacter arilaitensis*

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Abstract
Microbial Cellulase production is strongly influenced by factors such as temperature, pH, and incubation period. The present study evaluated the optimal culture conditions for cellulase synthesis by *Arthrobacter arilaitensis* isolated from horse dung. The strain exhibited maximum enzyme activity at 37 °C (0.0952±0.0057 U/mL), pH 7.0 (0.0644±0.0079 U/mL) and 72 hours of incubation (0.0921±0.0048 U/mL). Enzyme activity decreased significantly beyond these conditions. These findings confirm that *A. arilaitensis* is a mesophilic, neutral-pH-adapted cellulase producer, suitable for applications in ruminant nutrition and lignocellulosic biomass utilization.

Keywords: Cellulase, optimization, temperature, activity, *Arthrobacter arilaitensis*, pH, incubation period

1. Introduction
Cellulose, the most abundant organic polymer, contributes significantly to lignocellulosic biomass in ruminant diets especially in the form of grasses, straws, TMR etc. This cellulose is resistant to enzymatic degradation due to its crystalline structure and association with lignin and hemicellulose which thereby contribute to higher crude fiber and lower digestibility coefficient. Cellulase enzymes produced by microorganisms enhance fiber degradation and digestibility, thereby improving nutrient utilization in ruminants and hence can improve the milk yeild. Hindgut fermenters such as elephant and horses have a rich microflora of cellulolytic bacteria in the hindgut, these can be easily isolated from the dung of these animals and can be cultured aerobically to produce cellulase (Singh *et al*., 2015) [6]. *Arthrobacter arilaitensis*, an aerobic bacterium having cellulase-producing ability, has been isolated from elephant dung and is maintained and propagated at the Department of Animal Nutrition, College of Veterinary and Animal Sciences, Mannuthy. Optimizing the organism's cultural parameters is required to maximize enzyme yield for potential application in animal nutrition and bioconversion processes. This study is designed to determine the effects of temperature, pH, and incubation period on cellulase production by *A. arilaitensis*.

2. Materials and methods
2.1 Isolation of bacterial culture
Arthrobacter arilaitensis was isolated from elephant dung from elephant sanctuary at Punnathur kotta, Guruvayoor and maintained on Bushnell-Haas medium containing carboxymethyl cellulose (CMC) agar plates (Vaishnav, 2023) [8] Cultures were sub-cultured every 10-15 days to maintain viability. The organisms were later grown in enzyme production media containing the following components: CMC (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4$·7H2O (0.2), NH_4NO_3 (1.0), FeCl3·6H2O (0.05), CaCl2 (0.02) and yeast extract (5.0). The optimization of culture conditions was carried out in three phases.

2.2 Optimization of the temperature
The bacteria, Arthrobacter arilaitensis were incubated in Bushnell-Haas medium containing carboxymethyl cellulose (CMC) agar at 25 °C, 30 °C, 35 °C, 40 °C, and higher enzyme

activity was observed at 35 ℃ and 40 °C when compared with other temperatures and was similar. Hence, in order to find optimum temperature for the incubation, another incubation was done at 37 °C. (Islam and Roy 2018) [1].

2.3 Optimisation of the pH
The pH levels of the media were made to 5,6,7,8 and 9 using 1N HCl and 1N NaOH, and the activity was measured. (Latorre *et al*., 2015) [3].

2.4 Optimization of incubation periods
Incubation periods taken for the experiment are 24, 36, 48, 72, and 96 h., and the enzyme activities were noted Joseph *et al*., (2016) [2].

2.5 Estimating Enzyme Activity
After each incubation, cultures were centrifuged at 12000g for 20 minutes at 4 ºC, and the supernatant was used as crude enzyme. Carboxy Methyl Cellulase (CMCase) activity was measured using the dinitrosalicylic acid (DNS) method (Miller, 1959). In this method, 500µL of the crude enzyme was mixed with 500µL of 1% w/v carboxy methyl cellulose (CMC) in 50mM phosphate buffer and incubated at 37 ºC for 15minutes in a test tube. The reaction was stopped by adding 1mL DNS reagent and boiling for 10 minutes. The colour of the reaction mixture varied from light orange to dark orange depending on the amount of glucose released from CMC by the action of cellulase. The colour formed is then measured by a UV-VIS spectrophotometer at 540nm, and the concentration obtained is used to calculate the enzyme activity in U/mL. One unit (U) of cellulase activity was defined as the amount of enzyme releasing 1 µmol of glucose per minute at 37 °C, at pH 7.

2.4 Statistical anlaysis
The Data obtained from the experiment were analysed statistically as per Snedecor and Cochran (1994) by analysis of variance (ANOVA) technique, using the software Statistical Product and Service Solutions (SPSS) version 24.0.

3. Results and Discussion
3.1 Enzyme activity of cellulase isolated from *Arthrobacter arilaitensis* at different temperatures
The enzyme activity showed a significant temperature dependence, with the highest activity at 37°C (0.0952±0.0057). Beyond this point, activity declined (Table 1) suggesting thermal denaturation of the enzyme. These findings are consistent with Islam *et al*. (2019) [1], who reported peak cellulase activity of *Paenibacillus* spp., *Bacillus* spp., and *Aeromonas* spp. at 35 °C with a subsequent decline above 40 °C.

Table 1: Enzyme activity[1] of cellulase isolated from *Arthrobacter arilaitensis* incubated at six different temperatures

Temperature ºC	Activity U/mL
25	0.0305^{a}±0.0062
30	0.0490^{b}±0.0062
35	0.0798^{cd}±0.0039
37	0.0952^{d}±0.0057
40	0.0675^{c}±0.0062
45	0.0274^{a}±0.0041
P-Value	0.00*

[1]Mean values based on 6 replicates with SE
*Mean is significantly different from the test value (p<0.01)
[a-d] Mean of different treatments having different alphabets as superscripts between rows differ significantly (*-p<0.01)

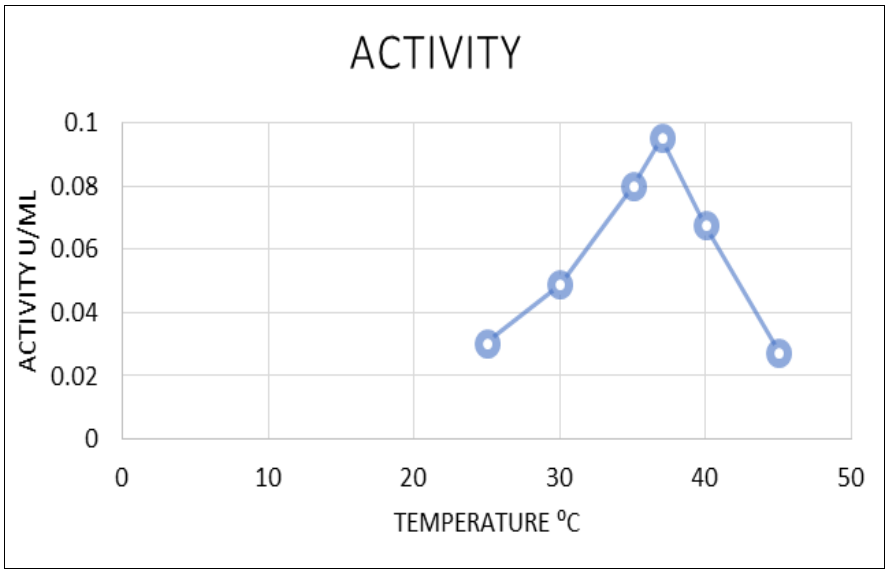

Fig 1: Enzyme activity of cellulase isolated from *Arthrobacter arilaitensis* incubated at six different temperatures

3.2 Enzyme activity of cellulase isolated from *Arthrobacter arilaitensis* at different pH levels
The highest activity was recorded at pH 7 (0.0644±0.0079), which was significantly higher (p<0.05) than other pH treatments. At pH 6 and 8, moderate activity was observed while extreme acidic (pH 5) and alkaline (pH 9) conditions yielded the lowest activity. The enzyme activity is depicted in Table 2. Similar results were reported by Joseph *et al*. (2016) [2], who found optimal cellulase production in Bacillus subtilis strains at near-neutral pH.

Table 2: Enzyme activity[1] of cellulase isolated from *Arthrobacter arilaitensis* incubated at five different pH levels

pH	Activity U/mL
5	0.0243[a]±0.0039
6	0.0397[a]±0.0057
7	0.0644[b]±0.0079
8	0.0397[a]±0.0057
9	0.0243[a]±0.0039
P-Value	0.000*

[1] Mean values based on 6 replicates with SE
*Mean is significantly different from the test value (p<0.01)
[a-b] Mean of different treatments having different alphabets as superscripts between rows differ significantly (*-p<0.01)

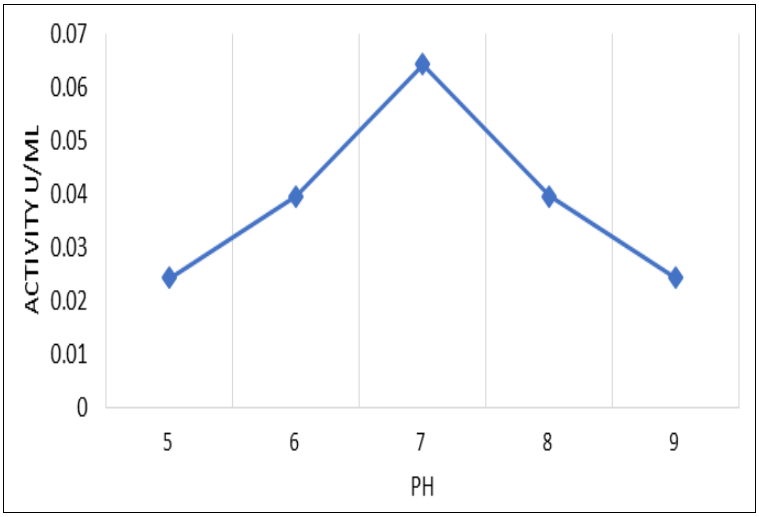

Fig 2: Enzyme activity of cellulase isolated from *Arthrobacter arilaitensis* incubated at five different pH, U/mL

3.3 Enzyme activity of cellulase isolated from *Arthrobacter arilaitensis* at different incubation periods

Enzyme activity progressively increased with incubation time, and highest activity was obtained at 72 h (0.0921±0.0048). Initial activity at 24 h was low, reflecting limited enzyme secretion. However, activity decreased after 72 h, with a reduced value at 96 h likely due to nutrient depletion or feedback inhibition. The activity at various incubation periods are given in Table 3. Joseph *et al*. (2016) [2] also demonstrated strain-specific variations in optimum incubation times, with 48, 72, and 96 hours for B. subtilis strains CB3, CB4, and CB8, respectively.

Table 3: Enzyme activity[1] of cellulase isolated from *Arthrobacter arilaitensis* incubated at five different incubation periods

Incubation period (hrs)	Activity U/mL
24	0.0305[a]±0.0039
36	0.0428[a]±0.0039
48	0.0675[b]±0.0039
72	0.0921[c]±0.0048
96	0.0706[b]±0.0057
P value	0.000*

[1] Mean values based on 6 replicates with SE
*Mean is significantly different from the test value (p<0.01)
[a-c] Means of different treatments having different alphabets as superscripts within a column differ significantly (*-p<0.01)

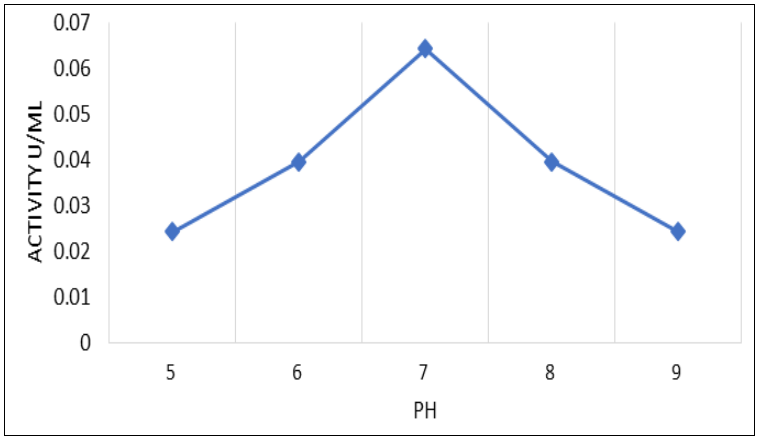

Fig 3: Enzyme activity of cellulase isolated from *Arthrobacter arilaitensis* incubated at five different incubation period, U/mL

4. Conclusion

The optimisation study demonstrated that Arthrobacter arilaitensis is a mesophilic, neutral-pH adapted cellulase producer with peak enzyme activity obtained at 37 °C, pH 7.0, and 72 h incubation. These parameters provide a scientific basis for scaling up cellulase production for applications in lignocellulosic biomass degradation and ruminant feed supplementation.

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Conflict of Interest: Not available

Financial Support: Not available

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