The Applied Biology & Chemistry Journal
https://www.theabcjournal.com
eISSN: 2582-8789
Original Research

Isolation, screening and optimization of cellulase production by a novel bacterial isolate of *Enterococcus durans*

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Received December 01, 2022
Revised December 27, 2022
Accepted January 04, 2023
Published February 05, 2023

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Abstract: Cellulose is an abundant plant biomass and a renewable source of energy in the ecosphere. The breakdown of cellulose occurs via the cellulase enzyme, which is commonly produced by microbes. This study aimed to optimize the fermentation parameters for enhanced cellulase production. Standardized parameters include isolation and screening of cellulase-producing bacteria (CPB), production of an enzyme, biochemical and molecular identification of bacterial isolate, optimization of cultural parameters, and application in wash performance. A total of 581 bacterial strains were isolated from soil samples, of which 16 isolates formed zones of hydrolysis on carboxymethylcellulose (CMC) agar media and were categorized as CPB. Based on maximum hydrolysis zone formation, three isolates, Krishi Vigyan Kendra-5 (KVK-5), Greenhouse-4 (GA-4), and Medicinal Garden-5 (MG-5) were chosen for bacterial cellulase production (BCP), with the isolate MG-5 proving to be the best cellulase producer (1.75 ± 0.01 U ml^{-1}). Based on 16S rRNA gene sequencing the isolate MG-5 was identified as *Enterococcus durans*. The optimized parameters for the production of the cellulolytic enzyme were an incubation period of 48 h, CMC (carbon source), and yeast extract (nitrogen source) at a concentration of 1.5% w/v, pH 7, 45 °C, 1.5% v/v inoculum size and 100 rpm. Optimum conditions resulted in a 1.92-fold increase (3.36 U ml^{-1}) in cellulase activity. Cellulase enzyme when used with detergent (Surf Excel), resulted in more efficient removal of chocolate stains on cotton fabric. This is the first report of *Enterococcus durans* producing cellulolytic enzymes. The analysis of cellulase in stain removal provides valuable evidence regarding the application of this enzyme in laundry cleaning.

Keywords: *Enterococcus durans*; isolation; screening; cellulase; wash performance

1. Introduction

In green plants, cellulose is the chief cell wall component and it is also secreted by numerous types of microbes like algae, yeasts, bacteria, oomycetes as well as fungi. Cellulose comprises thousands of carbon, hydrogen, and oxygen atoms and thus is categorized as an organic compound. Cellulose possesses properties such as insolubility in major organic solvents and water, is tasteless and odorless as well as is biodegradable [1]. The large amount of energy used by humans is derived from fossil fuels (non-renewable energy resources); however, a sharp decline in these energy resources is observed due to population growth that will result in the depletion of these resources. Hence, the shortage of energy resources due to the increase in fuel consumption can be controlled by the development and application of cellulose (renewable) resources. Cellulase performs a substantial part in the biological utilization of cellulose by causing its breakdown into fermentable sugars that can be used for biofuel production [2]. Cellulase has three enzymes-endoglucanase, exoglucanase, and β-glucosidase which act synergistically for the hydrolysis of cellulose. Cellulase occupies about 20% of the global enzyme market thus, making it an industrially significant enzyme [3].

There is an increase in the application of cellulolytic enzymes in diverse sectors such as the textile industry (biostoning, biopolishing, and bleaching of denim) [4]; paper industry (pulp processing and deinking); health (remedy for production of biofilms by *Pseudomonas* as a substitute to antibiotics) [5]; foods and beverages industry (processing and clarification of fruit juices); biotechnology (bioethanol production as well as valorization of lignocellulosic biomass); detergents and washing agents [4-6].

Presently, maximum cellulolytic enzymes (test site and marketable level) are procured from fungi: mostly *Penicillium*, *Aspergillus*, and *Trichoderma* because of their maximum enzyme activity and hydrolysis potential [7-9]. Nevertheless, BCP is more appealing because of its diverse nature as well as the higher growth rate of bacterial strains that produce them. Most notably, diverse bacteria occupy a broad range of ecological habitat and thus, helps in the selection of environmental stress-resistant cellulolytic strains [10]. Furthermore, enzyme production from these strains mostly possesses stability under harsh conditions.

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Citation: Bhati N, Shreya, Sharma AK (2022). Isolation, screening and optimization of cellulase production by a novel bacterial isolate *Enterococcus durans*. *T Appl. Biol. Chem. J*; 3(4):71-78. https://doi.org/10.52679/tabcj.2022.0008

Several bacterial genera are involved in extracellular cellulolytic enzyme production such as *Ruminococcus*, *Bacillus*, *Acetivibrio*, *Clostridium*, *Cellulomonas*, *Enterococcus*, and *Alteromonas* [9].

Lower enzyme yield is a major hurdle in the economic feasibility of the production process of cellulase, thus influencing the large-scale industrial application of this enzyme negatively. The drawback related to reduced enzyme yield can be controlled either by the use of appropriate microbial strains or through the optimization of physicochemical parameters associated with the fermentation process [11]. Extracellular production of cellulolytic enzyme can be monitored by the nutrient constitution of media like carbon source, and mineral salts as well as physical parameters like incubation time, pH, and temperature. These parameters perform an important part in the advancement of industrial bioprocess for enzyme production [12-16].

2. Experimental

2.1 Isolation of bacteria from soil samples

The diverse groups of microorganisms were isolated from soil samples collected from Krishi Vigyan Kendra (KVK), Medicinal Garden (MG) as well as a Greenhouse (GA) Banasthali Vidyapith (26° 24' 29.88" N 75° 51' 53.64" E), Rajasthan, India. A serial dilution technique was used for isolation and 100 µL of the suspension from the diluted sample was inoculated into nutrient agar media (NAM) for bacterial growth. Inoculation was performed under the sterilized environment and the plates were nurtured for 48 h at 35 °C for bacterial growth [17].

2.2 Screening of CPB

The cellulose-degrading activity of the bacterial isolate was analyzed by culturing on the CMC agar media supplemented with 1% w/v CMC [18]. After the media is solidified, CMC agar plates were inoculated with bacterial culture and then nurtured at 35 °C for 48 h. Ensuing incubation the plates were swamped with 0.1% w/v of Congo red and stored for 15 min then were replaced with 1M NaCl for 20 min to form an evident hydrolysis zone. Cellulase activity was analyzed by evaluation of the clear zone surrounding bacterial isolate on CMC media [19].

2.3 Production of enzyme

Bacterial cellulase production was carried out using modified Mandels and Reese [20] media containing 1% w/v carbon source (CMC) [7]. Inoculation of overnight grown bacterial culture was done and cultured at 35 °C for 48 h [21].

2.4 Enzyme assay

Total cellulase activity was determined by filter paper assay [22]. Determination of reducing sugar was carried out by using dinitrosalicylic acid (DNS) reagent [23]. One unit of filter paper assay was defined as the amount of enzyme releasing 1 micromole of glucose from filter paper per ml per min.

2.5 Biochemical and molecular identification

The bacterial isolate showing maximum cellulolytic activity was identified using a common bacterial system identification manual [24]. Morphological identification of bacterial strain was done by gram staining while, biochemical characterization was carried out using various tests like Voges-Proskauer (VP), oxidase, catalase, indole, citrate, as well as sugars (lactose, fructose, glucose, glycerol, maltose, galactose, mannitol) fermentation tests by standard methods [24]. The hyper-producer CPB isolate was sent to NCCS, Pune for 16S rRNA gene sequencing.

2.6 Optimization of cellulolytic enzyme production through one factor at a time (OFAT)

Factors affecting cellulase production by Enterococcus durans include incubation period (24-168 h), carbon source (CMC, lactose, xylose, glucose, sucrose, and starch), and nitrogen source (peptone, meat extract, sodium nitrate, yeast extract, urea, and ammonium chloride), the concentration of CMC and yeast extract (0.5-2.5% w/v), temperature (25-55 °C), pH (3-9), agitation speed (50-200 rpm) and inoculum volume (0.5-2.5% v/v) were used for optimization [25].

2.7 Wash performance analysis of cellulase

A cotton fabric was taken and cut into pieces (10*10cm) and each piece was stained with chocolate. The cotton pieces were dried and then were given four types of treatments which are: (a) distilled water (100 ml); (b) distilled water with 1% w/v detergent (Surf Excel) (99 ml + 1 ml); (c) distilled water with purified cellulase (99 ml + 1 ml); (d) distilled water with purified cellulase and 1% w/v detergent (98 ml + 1 ml + 1 ml). The above treatments were incubated at 35 °C in agitating conditions for 120 min. Cotton fabrics were then taken out, dried up, and observed for the presence of chocolate residuals. The cotton piece stained with chocolate and water-washed was regarded as a control [26].

3. Results and Discussion

3.1 Isolation of microorganisms

Isolation of microorganisms was carried out from the soil samples of Krishi Vigyan Kendra (KVK), Medicinal Garden (MG) as well as Greenhouse (GA) Banasthali Vidyapith, Rajasthan, India. In the current study, a total of five hundred and eighty-one bacterial isolates were obtained (table 1). Amongst all the samples the highest occurrence of bacteria was from the greenhouse soil sample. Soil comprises a varied range of cellulose-degrading microbes. Deejing and Dittamart, (2015) isolated 160 isolates of bacteria, 12 fungal isolates, and 7 actinomyces isolates from the soil, rotting wood, microbial activator, compost, and cow dung [27]. According to a study, 245 cellulolytic aerobic strains of bacteria were isolated from diverse conservation areas in the subtropics of China [21]. Saratale et al. (2012) isolated 4 bacterial strains from a forest area, in South Korea [9]. Li et al. (2020) reported the isolation of 10 bacterial isolates from Min pig manure [28].

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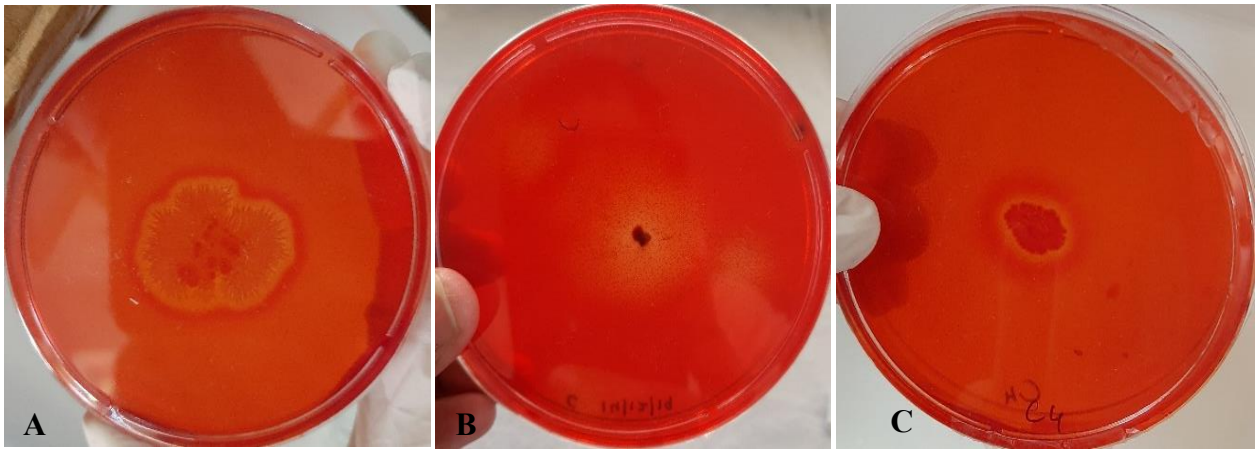

Figure 1. Zone of clearance produced by bacterial isolates; A) MG-5; B) KVK-5; C) GA-4.

Table 1. Isolation of cellulose-degrading bacteria from various soil samples.

Sources of soil sample	Number of bacterial isolates
Greenhouse	393
Krishi Vigyan Kendra	118
Medicinal Garden	70
Total	581

3.2 Screening of CPB

Congo red dye is mostly used for screening cellulase-producing bacteria. Sixteen bacterial isolates after Congo red dye staining showed a positive hydrolysis zone on the CMC plates (table 2). Extracellular cellulase secretion by microbes causes the breakdown of the cellulose present in their proximity thus, leading to the formation of a hydrolysis zone near the colonies as shown in figure 1. Isolate MG-5 displayed a maximum hydrolysis zone of 39 ± 0.4 mm. Li et al. (2020) screened 10 cellulolytic bacterial isolates for cellulose-solvent zones around colonies, among which the isolate M2 showed a maximum clearance zone (2.3 mm) [28]. According to a study, a cellulose-degrading zone (15.2 mm) was observed on the CMC plate, showing the cellulolytic activity of Bacillus sp. [11].

3.3 Cellulase production

The cellulolytic activity observed for the bacterial strain KVK-5, GA-4, and MG-5 was 0.98 ± 0.01 U ml^{-1}, 0.46 ± 0.01 U ml^{-1}, and 1.75 ± 0.01 U ml^{-1}, respectively, after 48 h of incubation (figure 2). The isolate MG-5 showed maximum activity thus, was selected for further characterization. Mahdi et al. (2010) reported maximum exoglucanase activity (1.13 U ml^{-1}) [29]. *Bacillus* sp. showed total cellulase activity as high as 0.14 U ml^{-1} [4]. A study reported that isolate ME27-1 showed the highest cellulase activity (0.17 U mL^{-1}) after incubation for 60 h in basal liquid media [21].

Table 2. Screening of cellulolytic bacteria.

Bacterial isolate	Hydrolysis zone (mm)	Bacterial isolate	Hydrolysis zone (mm)
KVK-10	28 ± 0.3	**MG-7**	22 ± 0.2
KVK-4	10 ± 0.3	**MG-5**	39 ± 0.4
KVK-5	32 ± 0.3	**MG-6**	17 ± 0.2
MG-4	20 ± 0.3	**MG-8**	12 ± 0.1
KVK-8	23 ± 0.2	**GA-2**	8 ± 0.3
MG-6	12 ± 0.3	**GA-9**	11 ± 0.1
GA-4	30 ± 0.4	**GA-10**	14 ± 0.2
GA-5	18 ± 0.1	**KVK-7**	10 ± 0.1

The isolates KVK, GA, and MG represent bacteria isolated from soil samples of Krishi Vigyan Kendra, greenhouse, and medicinal garden, respectively.

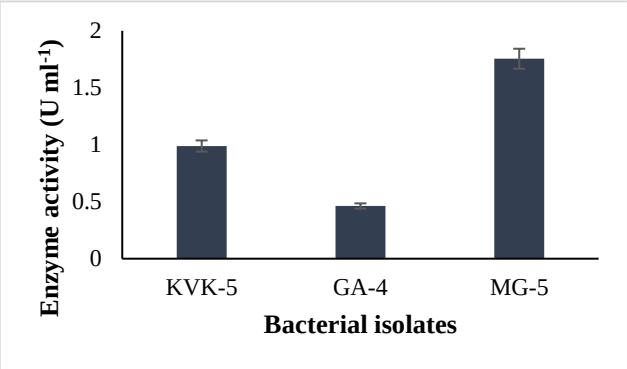

Figure 2. Cellulase production by bacterial isolates.

3.4 Biochemical and molecular identification

The biochemical characterization and molecular identification of isolate MG-5 are shown in table 3. The isolate MG-5 is Gram-positive, cocci-shaped, non-motile, and utilized sugars. The bacterial isolate MG-5 was identified by 16S rRNA gene sequencing, which showed a maximum identity of 100% with that of *Enterococcus durans* NBRC 100479(T), which has accession number OQ128114. The strains *E. faecalis* and *E. faecium* were isolated from the rumen of Tibetan yak, and their characterization revealed that both the strains were Gram-positive, catalase-negative, cocci-shaped, and showed sugar utilization [30].

Table 3. Biochemical and molecular characterization of isolate MG-5.

Characterization parameter	Results
Gram staining	+
Voges-Proskauer	+
Glucose	+
Fructose	+
Maltose	+
Lactose	+
Catalase	-
Citrate	-
Galactose	+
Mannose	+
Glycerol	-
Mannitol	-
Motility	-
Indole	-
Oxidase	-
16S rRNA gene sequencing	*Enterococcus durans*

"+" indicates positive; "-" indicates negative.

3.5 Optimization of fermentation parameters

Cellulolytic enzymes act as inducers, relying on the crux of substrate for their initiation and function. Fermentation parameters directly influence the microbial production of the enzyme. The enzyme production process must occur in media that is less costly and under the optimized condition on a large level to make the process cost-effective [31]. Thus, optimization of cultural conditions helps in making the production process of cellulase economically feasible.

3.5.1 Influence of incubation period

The incubation period in the production process plays a crucial role in cellulase production. The bacterial isolates were evaluated every 24 h for 7 days (figure 3). Maximum production of cellulase was observed after an incubation period of 48 h (1.84 ± 0.01 U ml^{-1}) and on the subsequent increase of fermentation time a decline in enzyme activity was detected (table 4). Cellulases are constituents of the primary metabolites, their production initiates at the logarithmic phase and diminishes at the death phase [32]. The optimum production of cellulase by *Paenibacillus terrae* occurs after an incubation period of 60 h which is much later than the incubation time observed for the strain *E. durans* [21]. The strain *Cellulophaga lytica* showed maximum cellulase production after an incubation period of 72 h [33].

Table 4. Influence of incubation period on BCP.

Incubation period (h)	Enzyme activity (U ml^{-1})
24	1.71 ± 0.02
48	1.84 ± 0.01
72	1.64 ± 0.01
96	1.46 ± 0.01
120	1.32 ± 0.01
144	1.05 ± 0.02
168	0.66 ± 0.01

3.5.2 Influence of carbon source

Carbon sources not only act as a bacterial source of energy but are also important inducers for the production of cellulase, as the microbes cannot directly assimilate these materials (cellulase) into their cells. Thus, the production of cellulases has been considered to be induced by soluble sugars [34]. In the present study, *E. durans* was grown on media having supplementation of various sources of carbon 1% w/v to induce cellulolytic enzyme production (figure 4A). Supplementation of CMC as a source of carbon exhibited the highest cellulolytic enzyme production (2.26 ± 0.01 U ml^{-1}) trailed by starch and sucrose. The optimum production of cellulase by *Bacillus wiedmannii* occurred in the presence of glucose as a source of carbon (0.0591 U ml^{-1}) [35]. *Arthrobacter woluwensis* isolated from soil showed the highest production of cellulase when CMC was taken as a solitary source of carbon [36].

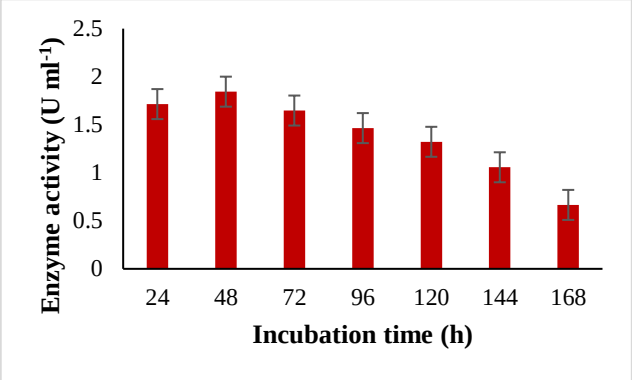

Figure 3. Influence of incubation period on BCP.

3.5.3 Influence of concentration of CMC

Varied CMC concentrations (0.5-2.5% w/v) were taken to attain maximum cellulolytic enzyme production (figure 4B). The optimum concentration of CMC was found to be 1.5% w/v (2.52 ± 0.01 U ml^{-1}) and a further rise in concentration led to decreased enzyme production. This decrease is associated with the fact that a rise in CMC (viscous compound) concentration increases the viscidity of fermentation media which impacts the controlled oxygen and nutrients circulation hence, results in a reduction in enzyme production. Comparable results were demonstrated by Das et al. (2022) having the optimal concentration of CMC being 1.5% w/v [36]. A study described the optimized CMC concentration for the production of cellulase by *Paenibacillus* sp. to be 1.0% w/v [37].

3.5.4 Influence of nitrogen source

Different sources of nitrogen were supplemented in the culture media (1% w/v) to study their impact on optimized cellulolytic enzyme production (figure 4C). Cellulase production was maximal in the presence of yeast extract (2.68 ± 0.02 U ml^{-1}) as a nitrogen source, followed by peptone (2.16 ± 0.01 U ml^{-1}). Several studies have shown that numerous organic and inorganic nitrogen sources, such as corn steep liquor [38], soya meal [39], and yeast extract [40], influence cellulolytic enzyme production. Organic

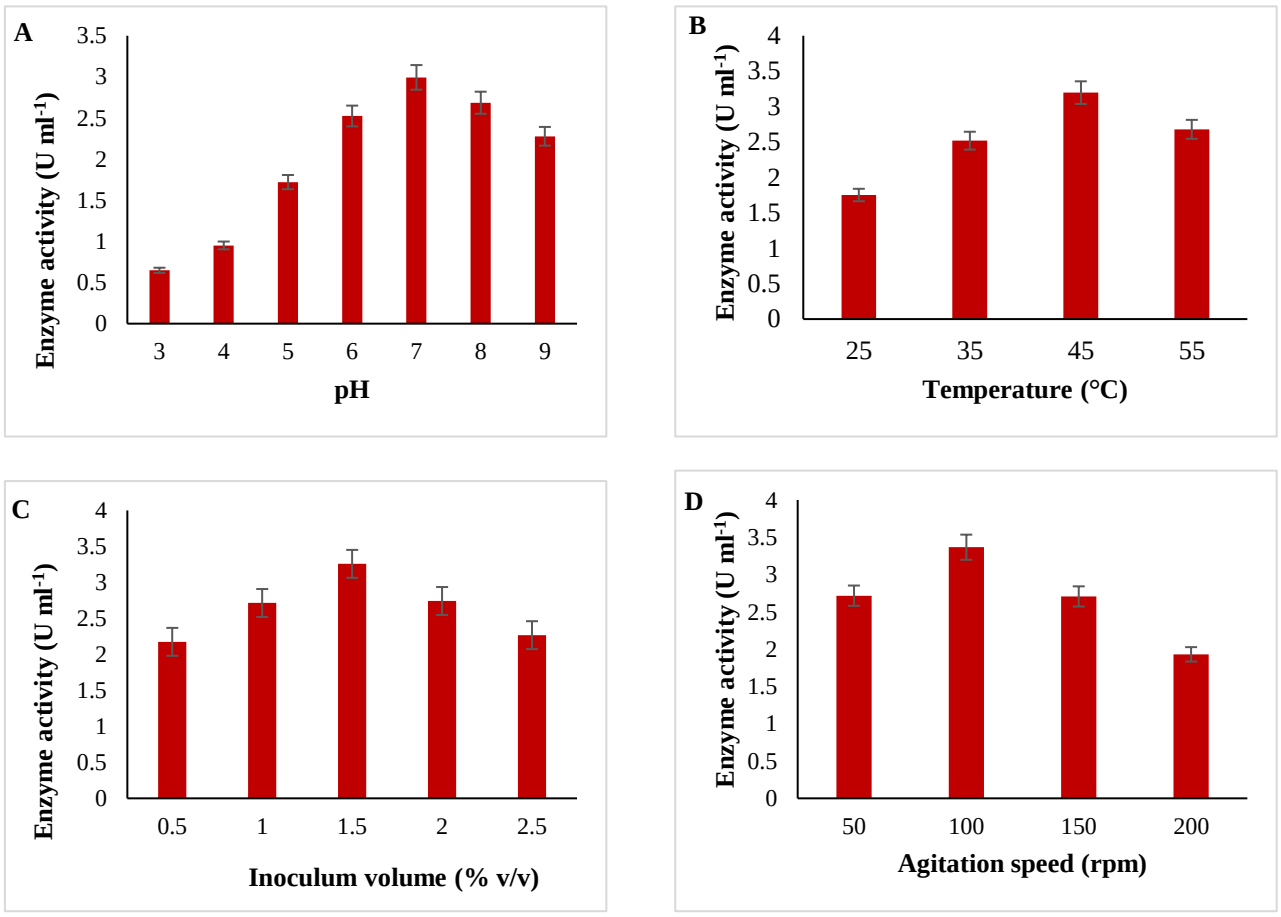

Figure 5. Influence of A) pH; B) temperature; C) inoculum volume; D) agitation speed on BCP.

nitrogen sources for instance yeast extract, result in the maximum production of cellulase [41].

3.5.5 Influence of concentration of yeast extract

Varied yeast extract concentrations (0.5-2.5% w/v) were supplemented in the culture media to achieve maximum production of cellulase (figure 4D). The optimal yeast extract concentration was detected to be 1.5% w/v (2.78 ± 0.02 U ml^{-1}). The highest cellulase production at 1.5% w/v concentration of yeast extract was reported [42].

3.5.6 Influence of pH

In the process of fermentation, the pH of the production media acts as a significant parameter that causes structural variations in microorganisms and enzyme release. pH fluctuations sensed in the process of microbial growth also affect the consistency of the product in the culture media. The optimal pH varies for numerous microorganisms as well as enzymes. The effect of different pH ranges (3-9) on the production of cellulolytic enzymes was determined (figure 5A). The highest cellulolytic enzyme activity was detected at pH 7 (2.99 ± 0.02 U ml^{-1}). Production of cellulase by *Bacillus velezensis* was optimum at pH 5 [28]. Pramanik et al. (2021) reported the optimum pH to be 7 for cellulase production by *Bacillus pseudomycoides* [1].

3.5.7 Influence of temperature

There is a direct influence of temperature on the growth, development, and metabolic activity of microbes. At low temperatures, there is no prospect of passing the substrate across the cell, which decreases the efficiency of cellulase production. Alternatively, at high temperatures, the energy required to sustain cell growth is higher because enzyme denaturation of the cell metabolic pathways decreases metabolite production. Studies indicate that the optimal temperature for cellulase production varies from species to species [43]. A different range of incubation temperatures (25-55 °C) was provided to study its effect on enzyme production (figure 5B). The highest cellulase activity was observed when the flask was incubated at 45 °C (3.19 ± 0.09 U ml^{-1}) and upon further increase in temperature there was a decrease in enzyme activity. *Bacillus wiedmannii* showed optimum enzyme activity at 60 °C [36]. Pramanik et al. (2021) demonstrated that the highest yield was attained by *Bacillus psudomycoides* at 40 °C [1].

3.5.8 Influence of inoculum volume

The concentration of bacterial strain inoculum has a direct influence on the production of cellulase. Varied inoculum volumes (0.5-2.5% v/v) were provided to fermentation media to study their effect on cellulolytic enzyme production (figure 5C). Low inoculum volume involves a large time for cell multiplication to adequate quantity for substrate utilization and enzyme production. Increasing inoculum volume would guarantee fast proliferation as well as biomass synthesis [44]. Optimal inoculum size was observed to be 1.5% v/v (3.25 ± 0.01 U ml^{-1}) for enhanced

cellulase production, and the subsequent rise in concentration led to reduced enzyme activity because of nutrient depletion, leading to decreased metabolic activity. Equilibrium between the growing biomass and available nutrients is crucial for the optimal production of the enzyme [44]. The optimal inoculum volume was 10% v/v for *Bacillus* sp. [44]

3.5.9 Influence of agitation speed

The impact of shaking speed on the production of cellulase was analyzed by using different agitation speeds (50-200 rpm) for incubation (figure 5D). The optimum agitation speed was observed to be 100 rpm (3.36 ± 0.02 U ml^{-1}). There is a direct influence of agitation on the rate of oxygen supply as well as the uniform distribution of nutrients and is important for the maximum production of the enzyme [45]. Though increased shaking speed may also cause high shear stress and have a negative influence on cell growth [46]. The optimum cellulase activity by *Bacillus* sp. at 150 rpm was reported [47].

3.6 Wash performance analysis of Cellulase

The effectiveness of cellulase as a detergent supplement was visibly analyzed. In this analysis, the chocolate stain was applied to cotton pieces and they were separately cleaned in an Erlenmeyer flask comprising distilled water, enzyme, detergent (Surf Excel), and detergent + enzyme combined. After the fabric was washed and dried, it was detected that the cotton fabric washed with a combination of detergent + enzyme was cleaned more efficiently than the cotton piece washed with detergent only. Shah et al. (2018) demonstrated the wash performance ability of the extracted cellulase on tomato sauce and turmeric paste stains and found the best results when the cloth was washed with cellulase + detergent [48]. A study reported that when the combination of chitosan immobilized enzyme and detergents was taken, chocolate stains removal on dirty fabric was more efficient [26].

4. Conclusion

A potential cellulose-degrading bacterial strain isolated from the soil was identified as *Enterococcus durans*. Biochemical characterization of the strain revealed its salient features, such as Gram-positive, cocci-shaped, and non-motile bacteria. The optimized conditions for the production of cellulase by strain MG-5 were: incubation period of 48 h, CMC (carbon source), and yeast extract (nitrogen source) at 1.5% concentration, 45 °C, pH 7, 1.5% inoculum volume, and 100 rpm agitation speed. Under an optimized environment, there was a 1.92-fold augment (3.36 U ml^{-1}) in cellulase activity in comparison to an unoptimized environment (1.75 U ml^{-1}). Optimization of fermentation parameters perform a very significant part in cost-effective enzyme production. Insight into the synergistic effect of the parameters on overall production positively contributes to the qualitative and quantitative production of enzymes from microbial sources and their application in modern biotechnology. Furthermore, the application of statistical modeling and computational biology for the optimization of process parameters can lead to improvement in product yield. Chocolate stain removal from fabric was more efficient with the combined use of cellulase and detergent. Hence, *E. durans* can be considered a potential source for cellulase production and can be explored further for its application in textiles, food, biofuel production, and the paper and pulp industries in the future.

Declarations

Author Contribution: Nikita Bhati performed investigation, experiments, result analysis and drafted the manuscript; Shreya performed the data and result analysis; Arun Kumar Sharma conceptualized and supervised the current research, and drafted, reviewed and edited the manuscript.

Funding: The authors declare that no funds, grants, or other financial support were received during this study.

Conflict of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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