



Sustainable Production of Bioethanol from *Bacillus Cereus* SD38 Using Raw Banana Peel Waste

Aamina Shahab¹, Syed Khalida Izhar¹, Shareen Fatima Rizvi¹ and Roohi Roohi^{2*}

1. Department of Biosciences, Integral University, Lucknow, Uttar Pradesh, India.

2. Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India.

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ABSTRACT:

Introduction: Increasing demand for renewable energy and sustainable waste management strategies has led to interest in agricultural residues as feedstock for bioethanol production. Banana peels, an abundant lignocellulosic waste, are rich in starch and represent a low-cost substrate for microbial bioconversion.

Objectives: The present study aimed to isolate amylase-producing microbes from soil samples and evaluate their potential to hydrolyze banana peel starch into fermentable sugars for subsequent bioethanol production using *Saccharomyces cerevisiae*.

Methods: Soil samples from the Lucknow region were screened for amylase-producing microbes using starch agar (primary screening) and amylase assays (secondary screening). Hydrolysis efficiency of the isolates was tested on chopped, fine-chopped, and powdered raw banana peels. The most effective strain, SD38, was selected to convert banana peel starch into glucose. Fermentation was carried out with *S. cerevisiae*, and ethanol was extracted using a rotary evaporator. Ethanol confirmation was performed by Jones and Iodoform assays.

Results: Among the isolates, strain SD38 exhibited the highest amylase activity and efficiently hydrolyzed banana peel starch. Fermentation of the resulting glucose by *S. cerevisiae* yielded ethanol, which was successfully confirmed through chemical assays.

Conclusions: Strain SD38 demonstrated strong potential in converting banana peels into fermentable sugars and bioethanol. This study highlights banana peels as a promising renewable and low-cost feedstock for sustainable bioethanol production, contributing to waste valorization and providing an alternative disinfectant source.

1. Introduction

Ethanol is a significant solvent for industries and raw chemical products for the production of medicines, cleaners, binders, plastics, and as a significant disinfectant (Matheus et al. 2024). Ethanol is being used as biofuel in combination with methane and hydrogen, replacing non renewable energy sources like coal and petroleum. Consequently, the production of bioethanol is currently regarded as one of the major commercial activities in several countries since it provides an alternative energy source [1]. Cellulose,

sugar, and starch are the three categories of agricultural raw materials that are conveniently employed in the fermentation process to produce bioethanol. Mineral acids are usually required to convert cellulose (wood, agricultural waste, and leftover sulphite liquor from pulp and paper mills) into sugars. Once simple sugars are generated, yeast enzymes may easily convert them to ethanol by fermentation. Furthermore, starch (grains, potatoes, root crops) must be degraded into fermentable sugars by the activity of enzymes from moulds [2]. The metabolic process of fermentation uses



microorganisms, typically bacteria or yeasts, in anaerobic environments to transform carbohydrates into alcohol or organic acids [3]. Research investigations are currently focused on two areas: the production of ethanol from cost-effective raw materials and the investigation of novel microbes or yeast strains that enhance the efficiency of ethanol production [4]. In this context, cost-effective raw materials including agricultural residues, cellulosic byproducts, fruit remnants, vegetable refuse, and industrial and municipal waste can be utilised for the economical production of ethanol. The enhancement of ethanol production via microbial fermentation relies on the selection of an optimal microbial strain, a suitable fermentation substrate, and appropriate procedure [5]. The biomass will undergo pre-treatment by several methods, including chemical, physical, and enzymatic treatments, to render the lignocelluloses available to the fermenting microorganisms [6]. Unripe green bananas are frequently thrown or used as animal feed. The genomic group of the banana plant is a significant component in determining its starch content. According to the International Network for the Improvement of Banana and Plantain (INIBAP, 2002), bananas are one of the most popular fruits consumed worldwide. Though banana peel is commonly discarded, it contributes about 30-40% of total fruit weight includes significant amounts of carbs, proteins, and fibre [7]. In some nations, the majority of its fruit peels are dried, powdered, and marketed to feed manufacturers at cheap prices. [8]. Despite their high carbohydrate content and other essential ingredients that can promote yeast growth, banana peels are an easily accessible agricultural waste that is underutilised as a possible growth medium for yeast strains [9]. Banana peels may be a suitable substrate for the synthesis of value-added products like ethanol because they contain lignin in trace amounts [10]. Ethanol is produced efficiently through optimised procedures that heavily depend on the use of inexpensive substrates. Banana peels are mostly a consequence of food industries, hotels, fruit markets, restaurants, and juice production facilities all over the world. The bioconversion of different agro-waste into ethanol has been the subject of extensive laboratory investigation. This work has focused less on banana peels and more on the sequential processes of pretreatment, hydrolysis,

fermentation, and distillation [11]. Thus, the purpose of this study was to produce value-added products (bioethanol) by utilizing banana peels by means of bacterial amylase and *Saccharomyces cerevisiae* enzyme.

2. Objectives

The primary objective of this study was to explore the potential of soil-derived amylase-producing bacteria for the sustainable production of bioethanol from agricultural waste, specifically raw banana peels. The work focused on isolating and characterizing potent amylolytic strains, with emphasis on identifying a high-performing candidate capable of efficiently hydrolyzing banana peel starch into fermentable sugars. Following microbial hydrolysis, the study further aimed to employ *Saccharomyces cerevisiae* to convert the liberated glucose into ethanol under optimized fermentation conditions. In addition, ethanol recovery and confirmation assays were performed to validate the process. Overall, this investigation sought to establish banana peels as a low-cost, renewable substrate for microbial fermentation, contributing to waste valorization and the generation of eco-friendly bioethanol with potential industrial and disinfectant applications.

3. Materials and methods

3.1. Soil sampling and primary screening of amylase producing bacteria

The sampling was done from the rhizospheric soil of a banana, potato and rice fields in Lucknow, Uttar Pradesh. Alpha-amylase producing strains were isolated by serial dilution using the spread plate approach. These samples were incubated at 37°C for 24-72 hours after being plated on a Starch Agar plate. The Gram's iodine solution was applied to these starch agar plates. The combination of starch and iodine results in a dark blue complex, and the bacterial growth in hydrolysed starch is surrounded by a distinct zone [12]. Potent bacterial strain was selected on the basis of zone of inhibition.



Table 1. Soil samples collected from different regions of Lucknow.

Sampling sites	Latitude/ Longitude	Total strains	Positive strains
Banana field of Basha Village, Lucknow, Uttar Pradesh	26.8140°N/ 82.7630°E	62	30
Rice field of Agriculture Department, Integral University, Lucknow	26.8465°N/ 80.94667°E	54	24

3.2 Secondary Screening of primary selected strains

The amylolytic activity of the primary chosen strain was evaluated by secondary screening with starch (Himedia) as a substrate. The approach described by [13] served as the basis for the assay technique. The amylase assay relied on the decrease in colour intensity that came from the starch being hydrolysed by the enzyme. 0.2 ml of enzyme (cell-free supernatant), 0.25 ml of 0.1% soluble starch solution, and 0.5 ml of phosphate buffer (0.1M, pH 6.0) made up the reaction mixture, which was incubated for 10 minutes at 50°C. 0.25 ml of 0.1N HCl was added to terminate the reaction, and 0.25 ml of Gram's iodine solution was added to develop the colour. A spectrophotometer set to 690 nm was used to measure the optical density of the blue colour solution. Units were used to express the activity. The amount of enzyme required to reduce the blue colour intensity of a starch-iodine solution at 50°C by 0.01% per millilitre in one minute is known as one unit of enzyme activity.

3.3 Tertiary Screening

A tertiary evaluation of selected strains was performed to determine their amylolytic activity using starch (Himedia) as a substrate. The research used the approach outlined by [13] to analyse the amylase enzyme generated by three different varieties of raw banana peel: chopped, finely diced, and powdered.

3.4 Morphological, physiological, biochemical identification of isolates

To determine the morphological, physiological, and biochemical traits of strain SD38 a thorough examination was carried out. The analysis of 16S rRNA gene sequencing was performed by Biokart Company in Bangalore, India. In this investigation, forward and reverse transcription were carried out using primer sets 27F and 149R, respectively. The BLAST search engine, which is a component of the NCBI database, was used to compare the nucleotide sequences included in the database with the sequences that were analysed. Nucleotide sequences were used to construct the phylogenetic tree, which was produced using MEGA X software [14].

3.4 Fermentation of raw banana peel using selected strain SD-38 under optimized conditions

Raw banana peel was used as the substrate for the fermentation process that produced bioethanol. 250 gm of substrate was combined with 500 mL of medium and autoclaved. SD38 and *Saccharomyces cerevisiae* were utilised as inoculum in medium because they catalyse the conversion of sugars into CO₂ and ethanol. The media was then incubated at 37°C for 1 month in static conditions. After one month of fermentation, media contained bioethanol and was used for further investigation.

3.5 Recovery of Ethanol

The produced ethanol was collected using a Buchi rotary vacuum evaporator set at 78 °C. In order to prevent heat-sensitive materials from degrading, a rotary evaporator uses a vacuum to decrease the boiling temperatures of solvents, rotation to increase the rate of evaporation, and condensation to recover the solvent. The obtained distillate was analysed for ethanol [15].

3.6 Qualitative test of fermented media and recovered bioethanol

The synthesis of bioethanol was examined using the Jones reagent in fermented and evaporated samples by K₂Cr₂O₇ + H₂SO₄ (Jones 1953). The Jones reagent was combined with 2 mL of sample, 5 mL of concentrated H₂SO₄, and 1 mL of K₂Cr₂O₇ (2%). The interaction of ethanol with potassium dichromate in the presence of sulphuric acid produced acetic acid with



a blue-green colour that indicates a positive test result [16].

4. Results

4.1.1 Primary screening of bacterial isolates

When the starch plates were flooded with a 1% iodine solution to test for amylase activity, a clear zone surrounding amylase-producing bacteria was seen in 31 isolates. Amylolytic activity was determined by evaluating the zone of clearance (Fig.1). Initially, the 15 bacterial strains were assessed according to their maximum hydrolysis zone. They showed a good zone of hydrolysis when flooded with iodine solution (Table 2).



Fig. 1. Starch hydrolysis test

Table 2. Primary Screening of microorganisms for amylolytic activity

S.No.	Diameter of colony growth (A) (mm)	Diameter of hydrolysis zone (B) (mm)	Ratio (B/A)
1.	10	11.2	1.12
2.	7.2	12	1.6
3.	5	14	2.8
4.	11.2	15.2	1.35
5.	6	12.4	2.06

6.	8.2	10	1.2
7.	8	16.4	2.05
8.	5	7.3	1.46
9.	6	14.8	2.4
10.	5	12.6	2.52
11.	12	18	1.5
12.	8	12.2	1.5
13.	6	16	2.6
14.	7.5	15.4	2.05
15.	4.8	15.2	3.1
16.	4.2	11.8	2.80
17.	11	13	1.18
18.	12.2	24.6	2.01
19.	7.4	11	1.48
20.	8	22.2	2.77
21.	6	18.6	3.1
22.	5	6.2	1.24
23.	3.2	10.4	3.37
24.	6.4	10.2	1.59
25.	3.8	10.6	2.78
26.	10.2	18.4	1.80
27.	4.8	10.2	2.12
28.	8	14.2	1.77
29.	5.2	18.4	3.53
30.	4.3	7.2	1.6
31.	6.4	18	2.81

4.1.2 Secondary screening of α -amylase

The primary chosen strain was subjected to secondary screening using starch (Hi medium) as a substrate in order to assess its amylolytic activity. Based on their amylolytic activity on starch, a total of five bacterial strains were selected for further investigation through secondary screening (Table 3).

**Table 3. Secondary screening of α -amylase**

S.No.	Strains	Activity of amylase enzymes (U/ml/min)
1.	Control	8200
2.	SC-18	7423
3.	SD-38	8980
4.	SC-18	5667
5.	SA-26	8454
6.	SA-36	6543
7.	SA-32	6212
8.	SA-15	8524
9.	SC-37	7945
10.	SA-22	6000
11.	SC-38	8528

4.1.3. Tertiary Screening

The amylolytic activity of the enzyme produced by the tertiary screening of chosen strains was evaluated based on a comparison of three different forms of raw banana peel: chopped, finely chopped, and powdered. Strain SD38 exhibits the highest activity with chopped banana peel (Table 4).

Table 4. Tertiary Screening of α -amylase with different forms of banana peel

S.No.	Strains	Amylase activity (U/ml/min)		
		Fine chopped	Chopped	Powdered
1.	SC-38	5164	6721	2077
2.	SD-18	3922	5715	1229
3.	SA-26	5447	6237	1695
4.	SA-15	4750	5815	1017
5.	SD-38	5834	7324	3517

4.2. Morphological, biochemical, physiological and phylogenetic analysis

The isolates were identified by studying morphological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology. Table 5 summarises all of the physical and physiological properties of the bacterial strain SD38.

Table 5. Morphological, biochemical and physiological analysis

Characteristics	SD38
Morphological tests	
Gram staining	Positive
Pigmentation	Yellow
Surface	Rough
Margin	Irregular
Cell shape	Rod Shape
Elevation	Convex
Biochemical Tests	
Catalase test	+
Amylase test	+
Casein hydrolysis test	+
Indole production test	-
Motility test	+
Methyl red test	+
Urease test	-
Acid production test (Maltose)	+



Acid production test (Dextrose)	+
Acid production test (Lactose)	-
Physiological Tests	
Optimum temperature for growth	37 °C
Optimum pH for growth	6.0-8.0
Growth at NaCl (%)	5-10

The SD38 strain's phylogenetic evaluation was completed with the alignment of a homologous sequencing of known bacteria and cladistics analysis, which classifies organisms according to their evolutionary relationships. The 16S rRNA sequences submitted to NCBI via Gen Bank (accession number PX205062) were identified using BLAST. According to its phylogenetic tree, which is shown in Fig. 2., the strain that was submitted to the NCBI was determined to be *Bacillus cereus*.

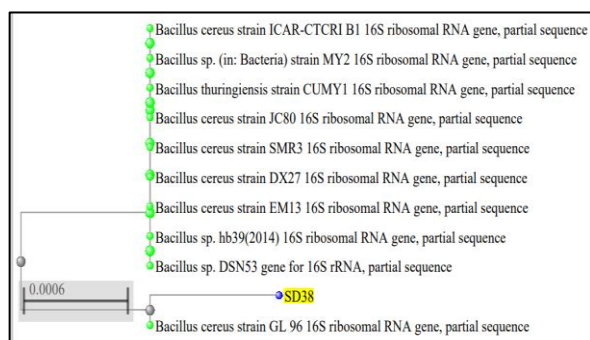


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequencing showing strain SD38 clustered with *Bacillus cereus*.

4.3. Qualitative analysis of Bioethanol

The Jones reagent was used to investigate the production of bioethanol in both fermented and evaporated samples using $K_2Cr_2O_7 + H_2SO_4$ (Jones 1953). Two millilitres of the sample, five millilitres of concentrated H_2SO_4 , and one millilitre of 2% $K_2Cr_2O_7$ were combined with the Jones reagent. With sulphuric acid present, the reaction between ethanol and potassium dichromate produced

acetic acid, which had a blue-green colour. A green colour indicates a positive outcome (Fig. 3).

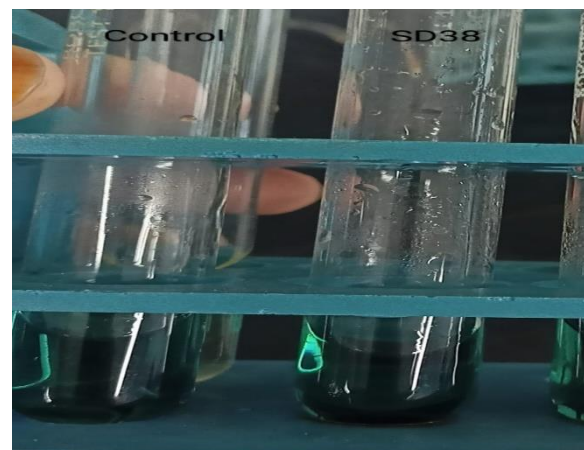


Fig. 3. Jones test of fermented media

4.4. Confirmation of Bioethanol by Iodoform Test

The iodoform test was used to confirm the purity of the bioethanol solution sample in both fermented and evaporated medium. One millilitre of the bioethanol sample and one millilitre of a 10% sodium hydroxide (NaOH) solution were placed in each test tube. A 10% iodine solution was gradually added, and it was heated for three minutes. Then, precipitates of yellow iodoform were found when it was compared to unfiltered fermented medium, the evaporated sample's iodoform test shows less yellow precipitate, indicating that it contains the highest proportion of pure ethanol.

4.5. Extraction of bioethanol from fermented media

A rotary evaporator was run thrice to extract about 20 millilitres of ethanol from the 500 millilitres of fermenting sample. In order to remove a solvent from a solution gently and effectively while maintaining a controlled temperature and lower pressure, this is repeated three times. In situations where gentle evaporation under vacuum is required to prevent thermal deterioration or loss of volatile components, it is especially well-suited for recovering volatile solvents like ethanol from solution (Fig. 4).



Fig. 4. Recovery of ethanol

5. Discussion

The findings of this study clearly demonstrate the potential of *Bacillus cereus* SD38 in the production of α -amylase and its application in bioethanol synthesis from raw banana peel waste. Primary and secondary screenings confirmed that SD38 possessed superior enzymatic activity compared to other isolates, with an activity of 8980 U/ml/min, while tertiary assays further highlighted its efficiency across different peel preparations. The higher activity observed with chopped banana peel (7324 U/ml/min) compared to powdered form (3517 U/ml/min) suggests that excessive processing may reduce enzymatic accessibility, a trend consistent with earlier observations on substrate pretreatment and particle size effects on enzymatic hydrolysis [17,18].

The morphological, biochemical, and molecular characterization of SD38 identified it as *Bacillus cereus*, a species previously reported for robust extracellular amylase production [19]. The alignment of its 16S rRNA sequence with closely related taxa in NCBI further confirmed this identity. Reports of *Bacillus* species exhibiting strong starch-hydrolyzing activity support the reliability of our results [20]. Moreover, the adaptability of SD38 to variable pH (6.0–8.0) and temperature (37 °C) strengthens its candidature for industrial fermentation settings, where process fluctuations are often inevitable.

Fermentation with *Saccharomyces cerevisiae* successfully converted the hydrolyzed sugars into ethanol, yielding ~20 mL from 500 mL of fermentation medium. The ethanol was confirmed using Jones and Iodoform assays, both of which are standard qualitative

methods for bioethanol verification [21]. The positive results underscore the feasibility of combining bacterial enzymatic hydrolysis with yeast fermentation as an integrated bioprocess for ethanol production. Such co-culture strategies have been proposed as cost-effective alternatives to chemical hydrolysis, especially when inexpensive agro-wastes are used as substrates.

Importantly, the valorization of banana peels into ethanol addresses two critical challenges: reducing agro-waste disposal and producing renewable biofuels. Banana peels, which account for 30–40% of fruit biomass, are commonly discarded despite containing high starch and carbohydrate levels. By demonstrating that banana peels can be efficiently converted into bioethanol without harsh pretreatments, this study contributes to sustainable waste-to-energy pathways. Compared with lignocellulosic substrates, which require extensive pretreatment to remove lignin, banana peels represent a simpler, low-cost alternative that can be integrated into decentralized bioethanol production systems.

Overall, this investigation establishes *Bacillus cereus* SD38 as a novel biocatalyst with high amyolytic potential, coupled with *S. cerevisiae* fermentation to produce ethanol. Future studies should focus on optimizing fermentation conditions, scaling ethanol recovery, and quantifying yields under pilot-scale conditions. In addition, exploring genetic or metabolic engineering approaches may enhance SD38's enzyme productivity, further improving process efficiency.

6. Conclusion

The study of α -amylase production by bacterial strain SD38, isolated from soil, demonstrates the significant potential of *Bacillus* species in industrial applications. Through rigorous screening, SD38 emerged as a high-performing candidate, exhibiting exceptional enzyme production under optimal conditions. This study focused on the pretreatment and hydrolysis of raw banana peel for the production of bioethanol using alpha amylase enzyme SD38 by fermentation using culture of *S. cerevisiae*. The presence of bioethanol in fermented media was confirmed by Jones test and Iodoform test. In comparison with existing literature, the study not only reaffirms the efficacy of *Bacillus* species in amylase production but also pushes the boundaries by successfully incorporating agricultural waste as a substrate, offering a scalable, eco-friendly alternative to



traditional methods. These findings present a significant advancement in microbial biotechnology, demonstrating that strain SD38 can be a key player in sustainable industrial processes.

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8. Declarations

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