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Characterization of Potential Enzyme - Producing Bacteria from Kitchen Waste

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Abstract: ***Bacillus subtilis is non - pathogenic gram - positive bacteria that has the potential to produce numerous organic substances of industrial importance. The present research work was focused on the isolation and partial characterization of soil bacteria that can produce amylase, cellulase, lipase, and proteases. This bacterial ability could further be utilized for the effective degradation of food waste generated from households and hotels. We find the bacterial isolate viz., KWB - 4 exhibited alpha - amylase, beta - amylase, protease, cellulase, and lipase activity. KWB - 4 was identified as Bacillus sp. that exhibited 97% similarity to Bacillus subtilis as per the morphological and biochemical characteristics. Further, several optimizations and activity enhancement approaches could be applied to B. subtilis (KWB - 4) to ensure the effective degradation of food wastes.***

Keywords: *Bacillus subtilis*, food - waste, enzyme activity, alpha - amylase, beta - amylase, cellulase, lipase, protease

1. Introduction

Bacillus subtilis (also called hay *bacillus*) is rod - shaped, gram - positive, and spore - forming bacteria. Most often these species are non - pathogenic and able to synthesize a diverse group of commercially potent extracellular enzymes [1]. *Bacillus* species are occupying a larger portion of the commercial enzyme groups [2]. The diverse group of enzyme synthesis viz., amylases, alkaline serine proteases [3], cellulases [4], lichenase, xylanases, β - galactosidase [5] have been observed with *B. subtilis*. These enzymes are well - known for their significant use in the food processing unit, serve as a digestive enzyme to improve intestinal health, as detergent additives to remove a tough stain, textile processing, leather softening, paper de - sizing, scouring, bleaching & pulp processing, and pharmaceuticals [6]. The Food and Drug Administration (FDA) includes *B. subtilis* under the Generally Recognized As Safe (GRAS). Thereby the enzymes such as amylase, cellulase, lipase, and proteases, which are produced from *B. subtilis*, can be used as additives and fortifiers in foodstuff [7]. The bacteria genera viz., *Acetobacter*, *Bacillus*, *Klebsiella*, and *Pseudomonas* have been frequently reported in soil and food waste [8], [9]. The present investigation was carried out to explore the extracellular enzymes produced by *Bacillus* species found in food waste.

2. Materials and Methods

Isolation of bacteria from Kitchen waste

The sample was collected from partially digested (ten days old fermented) kitchen waste. The ooze of fermented kitchen waste was collected and processed for bacterial isolation. The ooze was serially diluted to 10^{-7} and 0.2 ml of it spread over solidified Nutrient Agar Media (NAM) plates aseptically. The plates were then kept at 37±2°C for 48 hours.

Enzyme assay

The bacterial colonies appeared at the end of the incubation period. The pure culture of each bacterial colony was prepared by the streak plate method. Skim milk agar media, Tributyrin agar media, starch agar media and carboxymethylcellulose (CMC) agar media used for the qualitative protease [10], lipase [11], amylase [12] and cellulase [13] assay respectively. The qualitative enzyme assays were evaluated based on the appearance of a clear zone around the bacterial colonies at the end of the incubation period. Later, each pure bacterial culture was subjected to a quantitative enzyme assay. The Skim milk broth media, Tributyrin broth media, starch broth media, and carboxymethylcellulose (CMC) broth media were used for protease, lipase, amylase, and cellulase enzyme assay respectively. Each pure culture was inoculated in specified broth media separately and incubated for 96 hours. The incubated broth media were considered as crude enzyme extract and examined at every 24 hours of intervals for quantitative enzyme activity as mentioned below:

Alpha - amylase, Beta - amylase, Protease, Cellulase, and Lipase assay

***Alpha - amylase*:** The protocol adopted from Bernfeld (1951) for alpha - amylase activity assay.0.5 mL of starch (1%) in 0.02 M sodium phosphate (pH 6.9) mixed with 0.5 mL of crude enzyme incubated at 25°C for 10 min. The reaction mixture was placed into a boiling water bath (BWB) and 1 mL of 3, 5 - dinitrosalicylic acid (DNSA) was added to stop the reaction and filtered after 5 min. The clear reaction mixture was analyzed spectrophotometrically at 540 nm [14]. A glucose standard curve was made to calculate alpha - amylase activity.

***Beta - amylase*:** The protocol adopted from Bernfeld (1951) for beta - amylase activity assay [14].0.5 mL of starch (1%) in 0.016 M sodium phosphate (pH 4.8) mixed with 0.5 mL of crude enzyme incubated at 25°C for 10 min. The reaction mixture was placed into a boiling water bath (BWB) and 1 mL of 3, 5 - dinitrosalicylic acid (DNSA) was added to stop the reaction and filtered after 5 min. The clear reaction mixture was analyzed spectrophotometrically at 540 nm. A maltose standard curve was made to calculate beta - amylase activity.

***Protease assay*:** The protocol adopted from Ladd and Butler (1972) for protease activity assay [15]. Heat denatured (100^{o}C for 15 min at BWB) casein solution (1% in 0.05 M citrate buffer, pH 7.5) was mixed with 0.2 mL of crude enzyme and incubated at 37±2°C for 15 min.3.0 mL of trichloroacetic acid (10%) was poured into the reaction mixture and mixed well to terminate the reaction. The filtered reaction mixture was subjected to spectrophotometric analysis at 280. A tyrosine standard curve was made to calculate amylase activity.

***Cellulase assay*:** The protocol adopted from Miller (1959) for cellulase activity assay [16].0.25 mL of CMC (1%, pH 5.5) mixed with the 0.25 mL of crude enzyme and incubated at 37±2°C for 30 min. The 0.5 mL of DNSA was added to the reaction mixture and kept for 5 min in a BWB to stop the reaction. The filtered reaction mixture was subjected to spectrophotometric analysis at 540 nm. A glucose standard curve was made to calculate cellulase activity.

***Lipase assay*:** The protocol adopted from Yong and Wood (1977) for lipase activity assay [17]. by Olive oil was used as substrate. The 0.2 mL of the crude enzyme was dispensed into the test tube as well as a 0.2 mL of olive oil was added. The mixture was incubated at 40°C for 1 h. The reaction was terminated by adding absolute ethanol. The mixture was titrated with 0.1 M NaOH with phenolphthalein as an indicator. A change is observed when the mixture turns to pink. The values were read and recorded.

Characterization and identification of bacterial colonies

The potent bacterial strains which show the highest average enzyme activity were further subjected to characterization and identification. The gram staining and motility test were executed. The pure cultures of bacterial strain were inoculated to Difco sporulation medium (DSM) aseptically, inculcated at 37±2°C & 150 rpm for 48 hours [18], and then endospore formation was examined using Schaeffer - Fulton staining [19]. The colony and biochemical characterization were performed. The colony characters include shape, size, elevation, margin, surface, opacity, and color while biochemical characters comprise catalase, citrate utilization, gelatin hydrolysis, indole, MR (methyl red), VP (Voges Proskauer), nitrate reduction, urease, casein hydrolysis, esculin hydrolysis, tyrosine hydrolysis, gas, and acid production. Bacterial identification was done as per the key described by Sneath et al., (1986) and Reva et al., (2001) [20], [21].

3. Results and Discussion

In the present course of investigation twenty - three bacterial colonies were picked up from three master plates which were prepared from the samples of three sampling sites. Among them, six bacterial strains showed alpha - amylase and lipase ranges from 11.72 ±0.09 to 26.58 ±0.34 U/ml and 8.91 ±0.07 to 34.64 ±0.41 U/ml respectively, two strains beta - amylase ranges from 12.61 ±0.18 to 13.33 ±0.34 U/ml, five strains protease ranges from 13.54 ±0.27 to 38.54 ±0.46 U/ml, three strains cellulase ranges from 11.63 ±0.08 to 29.54 ±0.38 U/ml (Table No.1, Table No.2 and Fig.1.). Notably, KWB - 4 exhibited alpha - amylase, beta - amylase, protease, cellulase, and lipase activity. However, it exhibited lower beta - amylase (12.61 ±0.18 U/ml) activity than KWB - 7 (13.33 ±0.34 U/ml). Ciptaan and Ferawati (2019) reported 10.27 (U/ml) of protease and 17.13 (U/ml) of cellulose activity in fermented palm kernel cake by *Bacillus subtilis [*22]. Shart and Elkhalil (2020) observed 63.4 (U/ml), 41.2 (U/ml), and 28.3 (U/ml) lipase activity produced by *Bacillus* G14, *Bacillus* O1, and *Bacillus* B10 respectively [23]. Nusrat and Rahman (2008) reported the highest 4.5 (U/ml) of α - amylase activity in shake - flask cultures of *Bacillus subtilis* [24]. As the potent bacteria, in terms of enzyme activity, the KWB - 4 was identified as *Bacillus* sp. which showed 97% similarity to *Bacillus subtilis* as per the standard morphological and biochemical characteristics mentioned in the works of literature used for the identification (Table No.3.).

The potent microbial isolate investigated in the present research work could potentially be applied in the degradation of waste organic biomass generated from household waste and to convert them into manure simultaneously. Some literature divulged such kind of application earlier but the productivity was found to be lower. Barros et al., (2013) found ten *Bacillus subtilis* strains to exhibit amylase, protease, and lipase production [25]. An et al., (2018) assessed *Bacillus* species for the treatment of food waste and reported a 22.6% reduction in food waste after eight days of digestion at 45^{o}C [25]. Nivedhitha et al., (2020) isolated *Bacillus subtilis* and divulged that Mechanized Biological Treatment of kitchen waste was minimized up to 4.91 fold (from 20 kg to 4.07) in 16 days into manure [26]. Such a process reduces methane emission (as in the case of anaerobic composting occurs) and thereby helps to control global warming. A study conducted by Abbas et al., (2020) explored alkaline thermostable protease from *Bacillus subtilis* using skimmed milk [27]. Hence, the presently isolated bacterial strain could significanly be appicable for bio - waste degradation and support ecosystem by reducing global warming contribution.

Table 1: Qualitative assay of bacterial isolates: alpha - amylase, beta - amylase, protease, cellulase, and Lipase assay

Bacterial Strains	Alpha - amylase	Beta - amylase	Protease	Cellulase	Lipase
KWB – 1	-	-	+	-	+
KWB – 2	+	-	+	+	+
KWB – 3	+	-	-	-	-
KWB – 4	+	+	+	+	+
KWB – 5	+	-	-	+	+
KWB – 6	+	-	+	-	+
KWB – 7	+	+	+	-	+

Table 2: Quantitative assay of bacterial isolates: alpha - amylase, beta - amylase, protease, cellulase, and Lipase assay

Bacterial Strains	Alpha - amylase (U/ml)	Beta - amylase (U/ml)	Protease (U/ml)	Cellulase (U/ml)	Lipase (U/ml)
KWB - 1	-	-	13.54 ±0.27	-	16.15 ±0.31
KWB - 2	11.72 ±0.09	-	17.92 ±0.13	18.53 ±0.19	8.91 ±0.07
KWB - 3	19.43 ±0.16	-	-	-	-
KWB - 4	26.58 ±0.34	12.61 ±0.18	38.54 ±0.46	29.54 ±0.38	34.64 ±0.41
KWB - 5	14.63 ±0.12	-	-	11.63 ±0.08	-
KWB - 6	17.85 ±0.19	-	26.39 ±0.33	-	16.43 ±0.17
KWB - 7	24.58 ±0.34	13.33 ±0.34	23.48 ±0.31	-	19.82 ±0.28

Table 3: Colony and Biochemical characterization of KWB - 4

Morphological Characterization	
Colony color	Creamish - white
Size (colony diameter)	3.5 mm
Transparency	Opaque
Elevation	Flat
Margin	Round
Physical Characterization	
Gram Staining	+
Shape	Rod
Motility	+
Biochemical Characterization	
Catalase	+
Gelatin Hydrolysis	+
Indole	-
MR (Methyl Red)	-
VP (Voges Proskauer)	+
Citrate Utilization	+
Nitrate Reduction	+
Urease	-
Casein Hydrolysis	+
Esculin Hydrolysis	+
Tyrosine Hydrolysis	-
Gas	+
Acid from Glucose Fermentation	+
Acid from Glycerol Fermentation	+
Acid from Maltose Fermentation	+
Acid from Mannitol Fermentation	+
Acid from Starch Fermentation	+
Acid from Sucrose Fermentation	+
Acid from Xylose Fermentation	+

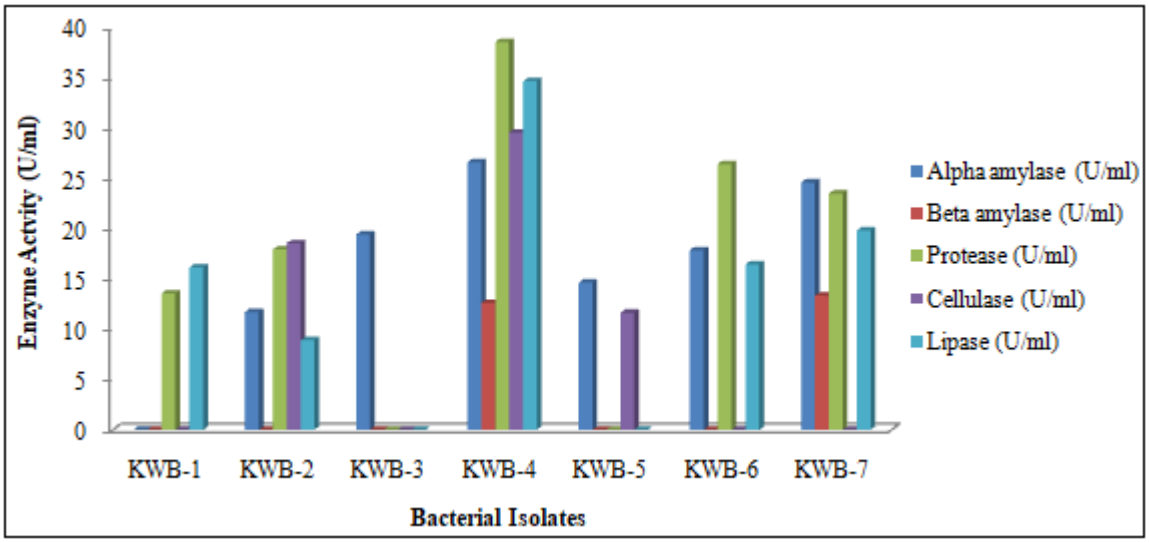

Figure 1: Quantitative enzyme activity of bacterial isolates. KWB - 1: Protease and Lipase activity; KWB - 2: Alpha - amylase, Protease, Cellulase, and Lipase; KWB - 3: Alpha - amylase activity; KWB - 4: Alpha - amylase, Beta amylase, Protease, Cellulase, and Lipase; KWB - 5: Alpha - amylase and Cellulase; KWB - 6: Alpha - amylase, Protease, and Lipase; KWB - 7: Alpha - amylase, Beta amylase, Protease, and Lipase.

4. Future Scope

The present work will further be extended towards the optimization of bacterial isolates for effective degradation of bio - waste. However some genetic modifcations must be needed for maximum output. But, the bio - augmentation might be open - up the way to immediate utilization of KWB - 7 for the degradation of bio - waste.

5. Conclusion

Presently we find the KWB - 4 has significant potency to produce an important group of enzymes. KWB - 4 was identified as *Bacillus* sp. that represents 97% similarity to *Bacillus subtilis.* The *B. subtilis* has well known as to serve diverse opportunities to go with genetic engineering, metabolic engineering, and synthetic biology - based modifications. It was widely explored to produce enzymes, organic substances, feed additives, biofilm, and so on. The desired - product - centric approach could be to *B. subtilis* using several optimizations and enhancement tools to ensure effective bioremediation applications.

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