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Production of Inulinase by *Bacillus* sp –recycling of agro waste using Banana peel, Garlic and Corn cob

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ABSTRACT

Inulinase production was evaluated by optimization of substrate and fermentation type. Two different fermentation with three different agro wastes were selected for this study. Inulinase producing bacteria was isolated and confirmed by qualitative congo red plate method. In order to determine the effect of agro waste such as corn cob, Garlic peel (GP) and banana peel (BP) on enzyme production substrate fermentation carried out and compared with submerged. Out of 5, higher extracellular inulinase was recorded in Bacillus sp and least inulainase activity noted in Pseudomonas sp. The production of enzyme by Bacillus sp under innulin enriched medium was less than (126U) banana peel (136 U) and garlic (130U) under submerged state. Compare to submerge substrate fermentation gave maximum innulinase activity and recorded as 146U and 122U respectively on BP and GP as substrate. The study on corn cob agro waste showed modrate enzyme activity at both state. The inulinase produced by the isolate have ability to withstand temperature up to 100°C. Hence the data concludes substrate fermentation (SuF) with banana peel is found to be ideal and good inulin substrate support for biotechnological production of inulinase enzymes under low cost. Further optimization of substrate concentration, pre treatments are needed to enhance the production of innulinase.

Keywords: Innulinase, Agrowaste, fructose, enzyme assay, fruit peel

INTRODUCTION

Agriculture creates huge amounts of waste, which poses a risk to human health, the environment, and animal health. To prevent and limit this danger, several waste treatment technologies are utilized. One of the primary goals of waste management strategies is to limit the amount of garbage disposed of in landfills and recycle organic stuff (Ahring, 2003). It comprises of mechanical pre-treatment, followed by an anaerobic or aerobic procedure to decrease waste effects. These procedures have received interest because they create stabilized waste that may be sold as fertilizer or disposed of in landfills, which will have the least impact on the environment (Adani *et al.,* 2003). Inulinases are enzymes that break down β-2,1glycosidic bonds, yielding fructose, inulo-oligosaccharides, and glucose. Exoinulinase extracts the terminal fructose units from inulin, yielding fructose as the primary product. Endoinulinase hydrolyzes inulin's intrinsic connections, resulting in inulo-oligosaccharides. Exoinulinases have both invertase and inulin hydrolytic activity, but endoinulinases do not (Vijayaraghavan *et al.,* 2009). Inulinases are often utilized in the manufacturing of ultra-high fructose syrup, ethanol, lactic acid, citric acid, and single-cell oil (Petrova *et al.,* 2015). Inulinase can also be used to produce bioethanol, citric acid, butanediol, and lactic acid. When the I/S ratio exceeds 10−2. It implies strong inulinase synthesis in culture, whereas it is less than 10−4 and indicates higher invertase production (Pessoni *et al.,* 2007).

Inulinase is produced by several bacterial and fungal species, including Streptococcus salivarius, Actinomyces viscosus, Kluyveromyces fragilis, Chrysosporium pannorum, Penicillium sp., and Aspergillus Niger (Chi *et al.,* 2009). A newly obtained Saccharomyces sp. from spontaneously fermented sugarcane produced inulinase when cultured on substrates such as banana peel, wheat bran, rice bran, orange peel, and bagasse (Onilude *et al.,* 2012). Coconut oil cake was utilized in a study to optimize the medium for inulinase synthesis using Pencillium rugulosum (Dilipkumar *et al.,* 2014). Sugarcane baggase and yacon have also been utilized as substrates for inulinase synthesis in several research (Chesini *et al.,* 2013, Mazutti *et al.,* 2006). Solid-state fermentation (SSF) mimics natural microbial processes such as composting and ensiling. Laboratory investigations are typically

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conducted in beakers, Roux bottles, jars, Erlenmeyer flasks, and glass tubes. Drum, deep tank, or tray fermentors have been employed to carry out large-scale fermentations (Mitchell *et al.,* 2006). Wheat bran, sugarcane bagasse, press mud, rice bran, garlic, onion peels, and other inexpensive, non-soluble substrates are the most typically employed for microbial inulinase synthesis. Non-soluble substrates have low thermal conductivity, which causes heat buildup and influences the creation of the final product (Singh and Chauhan, 2016). The fungal strains belonging to the Kluyveromyces (Mazutti *et al.,* 2006, 2007) and Aspergillus sp (Romero-Gómez *et al.,* 2000; Al-Dabbagh and Mahmood 2015) species are the most desired and often utilized for inulinase synthesis by SSF. The first report on inulinase synthesis utilizing garlic and onion peels by this approach came from the bacterial species Xanthomonas campestris (Ayyachamy *et al.,* 2007). Streptomyces species is an actinomycete that produces inulinase by SSF utilizing copra waste, pressmud, and garlic bulb powder (Dilipkumar *et al.,* 2011).

MATERIALS AND METHODS

Soil Sample Collection

Soil samples were gathered from agricultural land in Erode, Tamil Nadu, where the microbial activity is highest. Approximately 5g of dirt was collected using clean, dry, and sterile polythene bags and spatulas.

Preparation of Soil Suspension

To prepare soil suspensions, 1g of the obtained soil samples was dissolved in 10ml of sterile water.

Isolation of Bacteria

The isolation technique was carried out using nutrient agar medium. The soil suspensions were streaked onto nutrient agar plates and left to incubate for 24 hours at 37 degrees Celsius. Colonies were inspected and selected for future investigation.

Selection of Strain

A modified nutritional agar plate supplemented with 0.1% inulin was streaked serially with bacteria from agar plates. The medium's makeup was as follows: 0.1 g of inulin, 3.0 g of yeast extract, 5 g of peptone NaCl, 10.0 g of glucose, 4.0 g of agar, and 1.0 liter of distilled water with a pH range of 7.2-7.4 were incubated to see if inulinase was formed. An agar plate is dyed for five minutes with 0.1% Congo red before being placed in normal saline (0.85% NaCl) for fifteen minutes.

Screening of inulinase activity

The capacity of inulinase to hydrolyze inulin was used to test for its synthesis. The cultures were drenched with Lugos iodine and incubated for five minutes. After washing with water, the samples were incubated for 1 hour and tested for inulinase activity. The formation of a clear zone implies that a positive strain has been identified for further study.

Biochemical characterization of bacterial isolates

The morphological and physiological aspects of the isolates were studied using their colony characteristics on Nutrient agar medium and Gram's response. Following microscopic examination, the bacterial isolates were processed for identification using the usual recommended biochemical assays.

Gram’s staining

A tiny smear was formed, and then drenched with crystal violet. Gram's iodine was poured onto the smear, left for one minute, and then rinsed with water. The decolorizing agent alcohol was applied drop by drop until the dye was eliminated from the smear, and then rinsed with tap water. Finally, the smear was saturated with counter stain, safranin, allowed for 30 seconds, and then cleaned with water. The slide was air dried and examined under a microscope.

Indole Test

The bacterial culture was inoculated on tryptone broth and incubated at 37o C for 24-48 hrs. After incubation 0.5 ml of Kovac’s reagent was added and examined for the presence of cherry red colour in the upper layer of liquid.

Methyl Red Test

The bacterial culture was inoculated into MR-VP broth and incubated for 24-48 hrs at 37o C. After incubation few drops of methyl red reagent were added into the culture medium. Positive result was indicated by the formation of red colour and negative result was indicated by the formation of yellow colour.

Voges Proskauer Test

The isolated bacterial culture was inoculated into the MR-VP broth and incubated at 37^{o}C for 48 hrs. After incubation 0.3 ml of alpha napthol reagent and 0.1ml 40% KOH solution were added. Positive result indicated by the formation of strong red color and negative result was indicated by a no colour change.

Citrate Utilization Test

Simmon citrate slant was inoculated with the pure culture and the tubes were incubated at 37^{o}C for 24 hrs. After incubation, color change from green to blue color indicates positive result. Negative result was indicated by no color change. The medium remained green.

Catalase test

One drop of hydrogen peroxide solution was placed on a slide containing the culture. Vigorous bubbling occurring within 10 seconds indicated positive result. No bubble formation indicated negative result.

Oxidase test

Aseptically transfer a considerable amount of pure culture to the Oxidase disc. The disk was spotted for up to three minutes. If the region of injection changes from dark to maroon to virtually black, the outcome is good. If the color does not appear within three minutes, the outcome is negative.

Effect of substrate on enzyme production (Prabhjeet and Prabhjot, 2006)

Screening of strains for extracellular hydrolytic activities

The synthesis of extracellular hydrolases was detected using several enzymatic agar plate tests, with the exception of the inulinase activity assay, which was done in liquid medium. The pH of all media was adjusted to 7.2-7.4. The various assay medium utilized are explained as follows.

Pretreatment of Substrate

The corn cob, banana peels, and garlic powder are fully dried at 100°C for 72 hours. The dried slices were then ground into a fine powder using a hammer mill. Following milling, the resulting powder was directly employed as a carbon source.

Submerged Fermentation

Submerged fermentation was carried out at 37°C in 250 mL of 0.1% inulin supplemented with 1 g yeast extract and 0.5 g K2HpO4. 0.1% substrate extract was adjusted with 1 g yeast extract and 0.5 g K2HpO4. Without inulin, it is prepared. The broth was autoclaved at 121°C for 20 minutes. That flask was infected with 10 ml of 24-hour bacterial culture and incubated at 150 rpm for 24 hours, followed by 24 hours of shaking.

Substrate Fermentation (Bellon-Maurel *et al.,* 2003)

Substrate fermentation took place at 37°C in 250 ml of 10 g substrate, which included corn, banana peel, and garlic waste enhanced with yeast extract (1g), peptone (1g), dextrose (2g), and water (5 ml). The substrate water ration is 2:1. The whole flask was autoclaved at 121°C for 20 minutes. That flask was infected with 5 ml of 24 h bacterial culture and incubated at 150 rpm for 24 h and at rest for 24 hours.

Extraction of Inulinase

After fermentation, add 5 volumes of distilled water and agitate for 30 minutes at 200 rpm on a rotary shaker at 28°C. The material was centrifuged at 15000 rpm for 20 minutes. The supernatants were utilized to isolate proteins using ammonium sulphate precipitation and dialyzed for purity estimates and enzyme assays. The effect of temperature was recorded at 35, 50, 75 and 100°C for 10min incubation.

Assay of Enzyme Activity

Enzymes were tested by measuring the amount of reducing sugars produced from inulin or sucrose. We employed both culture filtrate and protein isolate. Incubate 1 mL of diluted crude enzyme with 4 mL of 2% inulin or 2% sucrose in 0.1 mM acetate buffer (pH 5.0) at 50°C. After 30 minutes of incubation, 0.5 mL aliquots were extracted. The rise in reducing sugar was calculated using a 3,5-dinitrosalicylic acid technique and a calibration curve using a reference fructose solution. Absorption was measured at 575 nm. A higher absorbance showed a higher degree of reducing sugar production, and hence a higher enzyme activity. One unit of inulinase activity (U) is the quantity of enzyme that produces 1 µmol fructose per min. The inulinase activity results were provided in

units of activity per gram of dry substrate (U/gds).

RESULTS AND DISCUSSION

Isolation and screening of inulinase producing bacteria

Bacterial colonies on nutrient-gar plates in seawater had 36 x 10^7 CFU (colony-forming units) per milliliter. It took 24 hours to document the bulk of the colonies. Colonies were classified as Erode bacteria 1-5 (EB1-5) based on morphology (Table 1).

Table 1: Colony morphology of colonies were categorized as Erode bacteria 1-5 (EB1-5)

CODE	COLONY MORPHOLOGY	Inulinase
EB1	White, small, irregular, opaque, convex, curved-shaped	-
EB 2	White, entire, circular, opaque, flat, curved-shaped.	Positive
EB 3	White, large irregular, opaque	-
EB 4	Creamy white, opaque small, irregular, opaque, convex, entire.	-
EB 5	White, translucent, large circular opaque, raised, curved-shaped	Positive

Using inulinase screening, five different colonies were selected and sorted. The Congo red-stained plate shows zones of substrate hydrolysis. Isolates that showed a clear zone on wheat bran agar plates were then transferred to inulin-containing agar plates. Only one of the five is found to be inulinase-positive. In terms of *Bacillus* sp. traits, isolate EB2 Gram positive endospore producing rod, MR, citrate, Catalase positive, and oxidase negative. During the primary screening, the five bacterial isolates from the *Bacillus* species reported in Table 2 showed higher hydrolytic zone development.

Table 2: Physiological properties of isolated *Bacillus* species

TEST	Eb1	Eb5
Gram stain	+rod	-rod
Indole	-	-
MR	+	+
VP	-	-
Citrate	+	+
Catalase	+	+
Oxidase	-	+

Production and assay of enzyme by *Bacillus* sp

The effect of fermentation in the substrate state is studied among positive isolates. *Bacillus* sp was inoculated into inulin-enriched broth, and fermentation was carried out for 48 hours. Corn cob, garlic, and banana peel were used as substrates. The enzyme content in the fermented extract was estimated using standard fructose. Figure 1 depicts the quantity of inulinase generated at different substrate of fermentation. Isolate *Bacillus* sp on substrate fermented medium produced enzyme activity. As a result, banana peel gave 146 U/mL, garlic gave 122 U/mL, and corn cob gave 88 U/ml. As a consequence, banana peel exhibited the highest enzyme activity.Banana peel substrate resulted in maximum production of inulinase earlier reported by Kuntal Kalra and Rashmi Kumari (2017). Recycling of agro waste such as wheat bran and corn steep liquor were found to produce therostable innulinase reported by Trivedi *et al.* (2012)

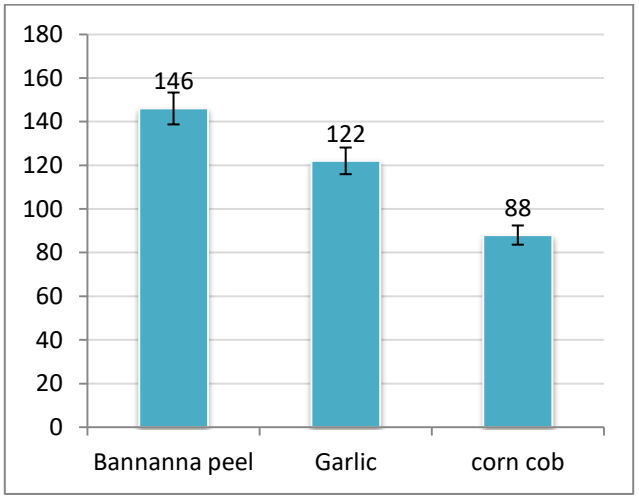

Figure 1: *Bacillus* sp on substrate fermented medium gave enzyme activity

Submerged fermentation

Similarly, extracts of submerged fermented media indicate 126 U/mL in Inulin, 80 U/mL in maize cob, 30 U/mL in garlic, and 136 U/mL in banana peel. The highest enzyme production was seen during substrate banana peel fermentation, followed by submerged fermentation with the same substrate (Figure 2).

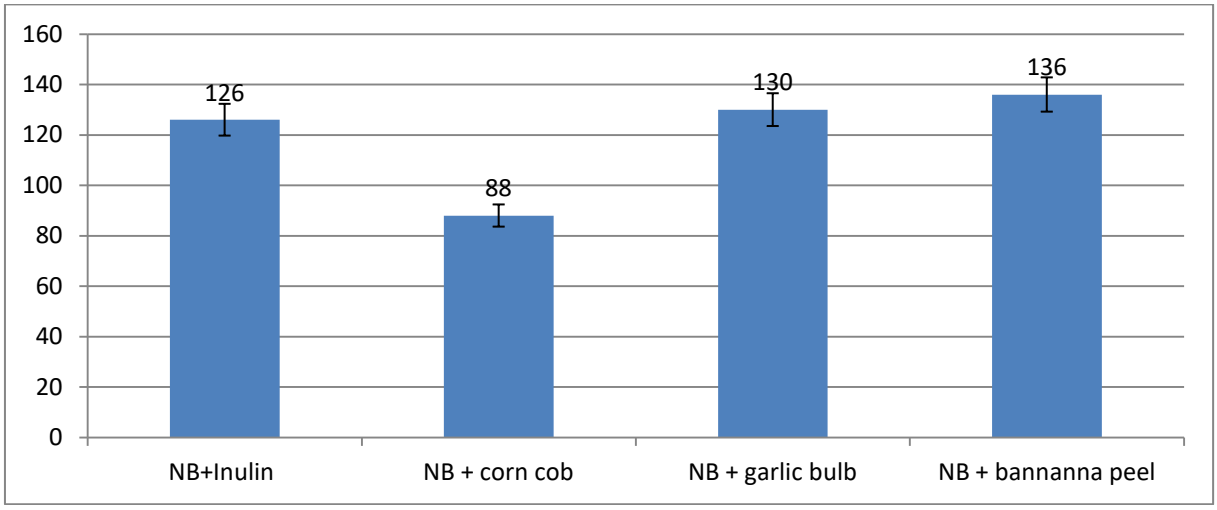

Figure 2: Effect of submerged fermentation on extracellular inulinase

Thermo stability of culture filtrate with enzyme activity evaluated at various temperatures, as shown in Figure 3, indicates that rising temperature, such as 37°C, 50°C, 70°C, and 100°C, lowered but did not stop activity, and was recorded as 120, 112, 98, and 88U. Previously thermostability of the enzyme was investigated and found best enzyme at 60 °C for 10 h (Gill *et al.*2006). Banana peel has been identified as an excellent fermentation substrate (Rehman *et al.,* 2014). Several researches were done to manufacture industrially relevant enzymes utilizing BP, including alpha-amylase by *Bacillussubtilis* and *Penicillium* species. (Akkarachaneeyakorn *et al.,* 2018).The main benefits of SSF over submerged fermentation include lesser moisture demands, greater yields, the opportunity to use renewable material, minimal investment, reduced energy requirements, better product recovery, and less environmental issues (Guerrero-Urrutia *et al.* 2021).

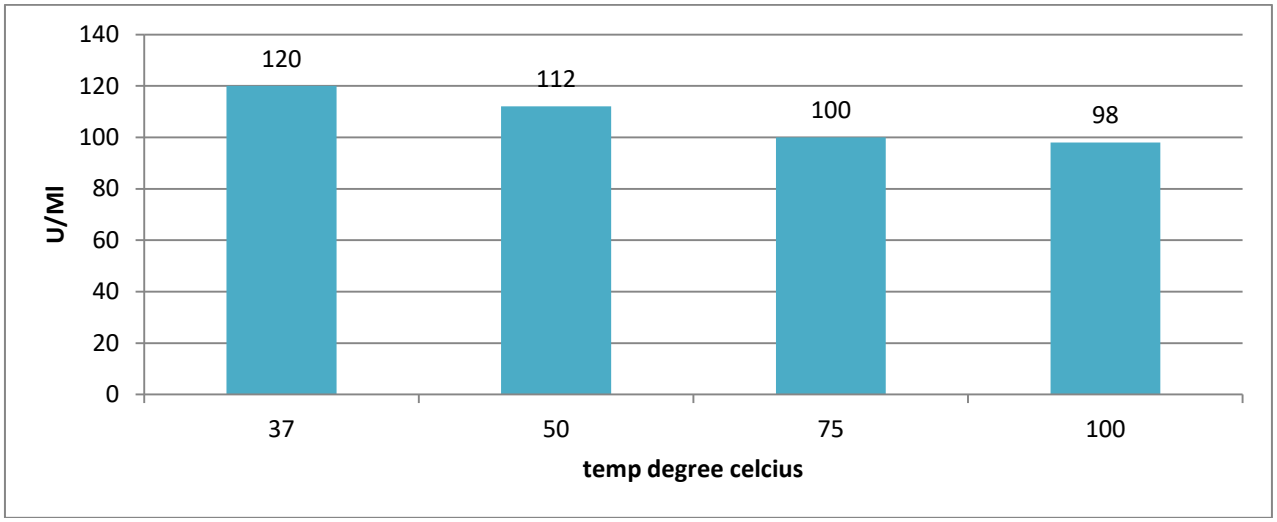

Figure 4: Effect of different temperature on inulinase activity

CONCLUSION

In this work, substrate fermentation was discovered to be efficient on inulinase synthesis by *Bacillus* sp using agricultural waste and may be perfect for maximal enzyme production.. Inulin and inulin-containing substrate are renewable, cost-effective polymeric carbohydrates that are easily hydrolyzed by microbial inulinases into fructose, glucose, and inulooligosaccharides. Furthermore, inulinase manufacturing from agricultural waste has the potential to be commercialized and used in both treatments and the food sector.

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