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Intracellular and Extracellular Fruit-Peel-Degrading Enzyme Synthesized by *Rhizobium* species CWP G34B

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This study investigated whether the fruit-peel-degrading (FPD) enzyme synthesized by the tropical *Rhizobium* species CWP G34B in the presence of some fruit peels was formed intracellularly or extracellularly. The *Rhizobium* spp. CWP G34B was grown in a minimal medium supplemented with 1% (w/v) pectin and peels of apparently healthy mature fruits: pineapple, orange, plantain, banana, pawpaw and mango separately. Dinitrosalicylic acid and Biuret reagent methods were used to quantify glucose and protein in the grown culture and the FPD enzyme activity was based on the amounts of the substances produced by the bacterium. Intracellular enzyme activity ranged between 0.01 mg.ml^{-1} glucose per mg.ml^{-1} protein (plantain peel) and 0.05 mg.ml^{-1} glucose per mg.ml^{-1} protein (pawpaw and banana peels). The extracellular enzyme activity ranged between 0.75 mg.ml^{-1} glucose per mg.ml^{-1} protein (banana peel) and 0.86 mg.ml^{-1} glucose per mg.ml^{-1} protein (pineapple peel). The total FPD enzyme activity (0.85 mg.ml^{-1} glucose per mg.ml^{-1} protein) was highest in pawpaw peel. The study showed that there was no significant difference between the extracellular and total enzyme activities of the FPD enzyme indicating that the FPD enzyme was majorly produced extracellularly.

Keywords: Fruit-peel-degrading enzyme activity, Intracellular and Extracellular synthesis.

The word enzyme, meaning 'in yeast' or 'leaven' was first coined and used by Kulme in 1876 to describe the catalytic action observed during fermentation when yeast was used to produce bread after mixing it with flour (Arotupin, 2007).

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Little was known about the chemical nature of enzymes before and during this time until the 20th century. Nevertheless, enzymatic reactions have been used by man since prehistoric time without the knowledge of the underlying mechanisms. The history of enzymology in the early part of the 19th century centres on the discovery that the extracts prepared from malt could convert starch to sugar and subsequently ferment the sugar to alcohol (Fawole, 1986). The potential of enzymes is employed in various metabolisms. Enzymes are involved in the metabolism of carbohydrates including the trehalose metabolism in microbes either in association with other organisms such as plants or when they are alone (Boboye, 2004). Cellulases, amylases, pectinases, proteases and lipases have been reported to be involved in the catabolism of cellulose, starch, pectin, protein and lipid among other substances.

Enzymes are sourced from plants, animals and microorganisms. Microbes have some advantages over plants and animals as sources of enzymes. Problems associated with plant and animal tissues are non-existence with microorganisms in the production of enzymes. Microbes require very little space for cultivation and large quantities of enzymes can be manufactured to meet the demand of the market within a relatively short length of time. More importantly, the diversity of enzymes available from microorganisms is so great coupled with their fast growth rates and the ease of enzyme recovery. In addition, microbial enzymes present a wide spectrum of characteristics that make them utilizable for specific applications (Boing, 1983).

Enzymes are produced by microorganisms in two ways. They are either secreted extracellularly into their immediate environment or intracellularly produced as integral part of the cells. Recently, we reported that *Rhizobium* species CWP G34B produced fruit-peel-degrading (FPD) enzyme (Boboye & Ajayi, 2011). This enzyme has the potential for commercial application in the fruit juice manufacturing industries. In this article, we investigated whether this non-pathogenic tropical bacterium synthesizes the FPD enzyme intracellularly or extracellularly. The project also examined whether the polygalacturonase formed by the rhizobium is inducible or not.

MATERIALS AND METHODS

Preparation of Fruit Peels and Test Bacterium

Nutrient broth powder was supplied by Lab M., Topley House, England. It was prepared according to manufacturer's specification (28 g/L). Minimal medium was prepared by dissolving 1.0g of disodium hydrogen phosphate (Na_2HPO_4), 0.2g of potassium chloride (KCl), 0.2g of magnesium sulphate ($MgSO_4$) in 1 litre of distilled water. All the media were sterilized by autoclaving at 121°C for 15 minutes.

Different fruits from which the peels were obtained for use in this study were purchased from "Oba", "Isinkan", "Iloro" and "Mojere" markets in Akure, Nigeria. The fruits were pineapple, orange, plantain, banana, pawpaw and mango. They were thoroughly washed in tap water and peeled using sterile kitchen knife. The fruit peels were sun dried between 30°C and 40°C for 5 hours daily for 7 days. Then, the peels were ground using Marlex Electroline Blender and kept in a refrigerator maintained at 4°C.

Mannitol, pectin and the test bacterium (*Rhizobium* species CWP G34B) were provided by the Department of Microbiology at the Federal University of Technology, Akure, Nigeria.

Test for Synthesis of Fruit-Peel-Degrading Enzyme and Polygalacturonase by *Rhizobium* species CWP G34B.

The microbe (18 hours old) was inoculated into 20 ml minimal medium (MM) containing 1% (w/v) of each fruit peel and pectin separately. They were incubated at 28°C for 24 hours. Polygalacturonase induction was conducted by growing the rhizobium in the MM supplemented with 1% (w/v) pectin and mannitol separately.

Extraction of Enzyme

Two and a half milliliters of toluene were added to 10 ml of the 24 hours old culture, mixed and left for 10 minutes at 28°C. Each culture, with and without toluene, was centrifuged at 3600 rpm for 15 minutes and used as sources of total and extracellular enzyme. The enzyme obtained from the toluene treated culture was total enzyme and the enzyme obtained from untreated culture was extracellular enzyme. In the pectin, the enzyme was named polygalacturonase.

Enzyme Assay for Fruit-Peel-Degrading Enzyme

Dinitrosalicylic acid (DNSA) and Biuret reagent methods were employed for the enzyme assay. Prior to this assay, Dinitrosalicylic acid (DNSA) reagent method (Bernfield, 1955) as modified by Boboye and Alao (2008) was used to prepare a glucose standard curve thus: the DNSA reagent (0.5 ml) was added to various concentrations (0 to 9.0 mg/ml) of glucose and left at room temperature (28°C) for 15 minutes. Then, each mixture was boiled for 5 minutes and cooled down with tap water. Four and a half milliliters of distilled water was added, cooled and the absorbance was read at 540nm. This set of values was used to plot a standard glucose curve. Biuret reagent method (Gornall *et al*., 1949) as described by Boboye and Alao (2008) was used to draw the protein standard curve using egg albumin prepared in the concentrations of 0 to 9.0 mg/ml. Biuret

reagent (0.5ml) was added to each concentration of the egg albumin and left at room temperature (28°C) for 15 minutes. The absorbance was read at 540nm and used to plot a protein standard curve. Quantities of glucose and protein formed in the enzyme sources were determined following the same procedures as described for the standard curves. The absorbance readings of the test samples were referred to the standard curves to estimate the corresponding glucose and protein values. The FPD enzyme activity was defined as the amount of glucose (mg/ml) per mg/ml of protein produced under this assay condition.

Assay for Polygalacturonase Activity

Induction of polygalacturonase activity was measured using the Thiobabituric acid (TBA) reagent method according to Boboye and Shonukan (1993). Three and half milliliters of 1% (w/v) pectin, 1mM calcium chloride and 1ml of the enzyme source were mixed and incubated at 35°C for 1 hour. The acid TBA reagent (3ml) was added to each mixture to stop the reaction. The control mix was the same as the experimental tube except that the enzyme source (supernatant) was substituted with the minimal medium. Both experimental and control reaction mixtures were boiled for 20 minutes, cooled rapidly under tap water and the absorbance was read at 540nm to estimate the amount of pectin that was degraded. One unit of polygalacturonase activity was defined as OD_{540} equivalent to mg/ml glucose produced from pectin by the polygalacturonase under this assay condition.

RESULTS

Intracellular and Extracellular Synthesis of Fruit-Peel-Degrading Enzyme and Polygalacturonase

The *Rhizobium* spp. CWP G34B synthesized fruit-peel-degrading (FPD) enzyme and polygalacturonase (PG) into each fruit peel and pectin used in this experiment. The total FPD and PG activities ranged between 0.75 (banana peel) and 0.85 $mg.ml^{-1}$ glucose per $mg.ml^{-1}$ protein (pawpaw and orange peels) (Fig. 1). Generally, FPD enzyme and PG activities were higher outside the cells than within the cells. The highest FPD enzyme activity expressed in the medium before the cells were lysed was 0.86 $mg.ml^{-1}$ glucose per $mg.ml^{-1}$ protein in pineapple peel while the lowest activity was 0.75 $mg.ml^{-1}$ glucose per $mg.ml^{-1}$ protein in banana peel (Fig. 1). There was no significant difference in the FPD enzyme activity between the lysed and unlysed cells when the bacterium was grown in the pectin and peels of mango, orange and plantain. Intracellular activity of the enzymes (FPD and PG) was 0.01 $mg.ml^{-1}$ glucose per $mg.ml^{-1}$ protein in plantain peel and pectin respectively. The intracellular FPD enzyme activity was 0.05 $mg.ml^{-1}$ glucose per $mg.ml^{-1}$ protein in pawpaw and banana peel each. In pineapple peel, the extracellular FPD enzyme activity was higher than the total FPD. In contrast, pawpaw and banana peels showed higher total FPD enzyme activity than extracellular enzyme activity.

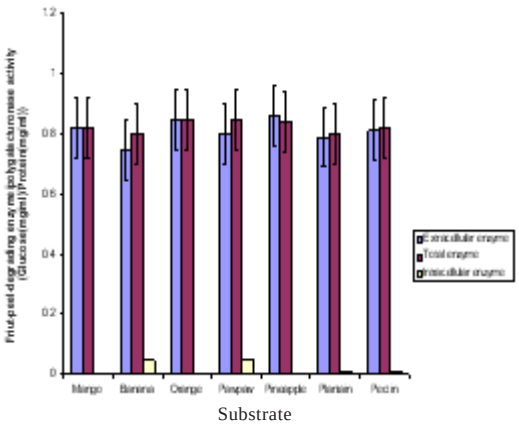

Fig. 1. Intracellular and extracellular fruit-peel-degrading enzyme and polygalacturonase activities

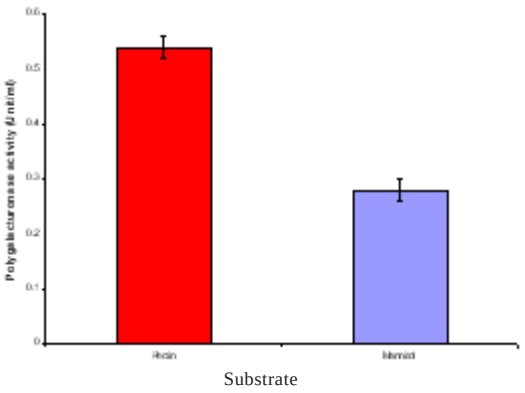

Fig. 2. Pectin catabolic enzyme synthesized by *Rhizobium* species CWP G34B

Induction of Polygalacturonase (PG) Activity of *Rhizobium* species CWP G34B

The *Rhizobium* spp. CWP G34B showed higher activity of polygalacturonase when grown in the minimal medium containing pectin than mannitol as shown in Fig. 2. The mean polygalacturonase activity was 0.54 Unit/ml in pectin in contrast to mannitol (0.28 Unit/ml).

DISCUSSION

The enzyme was constitutively synthesized with or without pectin. Lower PG expressed by the *Rhizobium* when grown in the presence of mannitol than in pectin means that pectin is an inducer of polygalacturonase and mannnitol could repressed the synthesis of the PG. The expression of polygalacturonase activity (PG) activity implies that the bacterium could breakdown fruit peels that are majorly made up of pectic substances, hence the activity of FPD measured in this experiment

Lack of significant difference between the extracellular and total enzyme released into the medium showed that the enzyme was majorly produced extracellularly. Enzymes secreted extracellularly are much easier to harvest than those secreted intracellularly. Therefore, less fund will be required to purify the enzyme than would be needed if chiefly secreted intracellularly. Also, the ability of the tropical *Rhizobium* to produce the enzyme extracellularly suggests that the purification of the enzyme may be less laborious for use in clarification of fruit juices and removal of fruit peels as cell lyses would not be necessary. However, the total activity of the FPD enzyme was higher than that of the extracellular enzyme made in banana and plantain peels; implying that intracellular FPD enzyme was made by the *Rhizobium*.

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