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Effect of Synthesized Biopolymer on Physiological and Biochemical Changes in Maize Seeds

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Abstract

The present study was conducted to check the mixed fruit waste as a substrate for biopolymer production. The hydrolysate obtained after alkali treatment was fermented by a novel strain *Enterobacter cloacae* and isolated bacterial strain (PS1 & PS2) to produce biopolymer. Ammonuim sulphate (1g/L) was a suitable nitrogen source for biopolymer production among the different nitrogen sources tested. The optimum environmental conditions were: initial pH 7.0, temperature 37 °C, and the C/N ratio was 20:1. Under these controlled conditions, biopolymer yield from *Enterobacter cloacae,* PS1and PS2 was elevated to 8.4,6.2 and 4.2 g/L, respectively. The FT-IR spectra of biopolymer from Isolate PS1 and PS2 indicate symmetric (O-C-O) presence at 1600cm^{-1} and asymmetric absorption peak at 1400 cm^{1}. The obtained results suggest that these biopolymers may be similar to PHB. The effect of biopolymer (BP 1) on physiological and biochemical changes in maize seedling growth at different NaCl salt stress concentrations (100, 200 and 300 mM) and Na_2CO_3 salt stress concentration (25, 50 and 75 mM) were studied. The values of starch content, protein content, alpha-amylase and protease activity were obtained to be higher at 0.4 to 2.4 % concentrations of BP1 when compared to control, Crabshell Extract (CE) and Green Polymer (GP) treatments.

Keywords: Biopolymer, seed priming, salinity, biochemical effect, amylase and protease

Introduction

Biopolymers are gaining a lot of interest and are considered appropriate substitutes for petroleum-based plastics. It is used for various applications in the medical, agriculture, and packaging fields. The consumption of petroleum-based plastics creates a severe disposal problem in landfills due to the slow rate of degradation [1]. Natural and synthetic biodegradable plastics are obtained from various sources; biodegradation of plastics is done through the chemical or enzymatic reaction associated with living organisms in the environment. The biodegradability of plastics depends on their chemical structure and degrading environmental conditions. Biopolymers including chitosan, wheat gluten, polyhydroxy-butyrate (PHB), starch and collagen, have all applications in agriculture, cosmetics, food and medical industry [2].

Enhancing the biopolymer production to get a high yield depends on the parameters with strain type, nitrogen and carbon sources, the reactor's volume, and cultivation time with high conversion rates [3]. A viscous strain possessing the highest flocculating activity was chosen to produce biopolymer and identified according to Bergey's Manual of Determinative

Bacteriology. From previous studies, the newly isolated *E. cloacae* WD7 was found to produce exopolysaccharide (EPS), possessing high flocculating activity with the EPS yield [3, 4]. Seed coatings of synthetic or natural polymers enhance seed germination, early seedling growth and yield under a stressed environment. It may also offer a protective barrier against bacterial contamination and spoilage [4].

The germination percentage is decreased with increasing salinity, attributed to the reduced water uptake and reduced enzyme activity. It modifies the enzyme activity in the rice endosperm such as amylase, protease and ribonuclease. These enzymes help to hydrolyse the complex food reserves for growing seedlings. Increasing salt concentration reduced seed emergence rate and seedling length during the germination of seeds. Numerous physiological processes such as starch and protein mobilization, nitrogen fixation, photosynthesis and root respiration are also affected under saline conditions leading to reduce crop productivity [5].

Thus, it is essential to study the influence of salinity on certain enzymes' activities during the plant's germination and growth. Therefore, the use of seed coating with synthesised polymer against NaCl and Na_2CO_3stress for improving the maize seed germination was investigated.

Materials and Methods

Bacterial isolation and screening: *E. cloacae* 811101 strain was procured from the National Institute of Agrobiological Science, Japan and it was used for biopolymer production. The microbes used in this study were isolated from sugarcane field soil to screen biopolymer-producing bacteria. Nine colonies from soil samples were selected and grown in nutrient broth and then preserved for the rest of the experiment. The isolated bacterial sample was qualitatively screened for biopolymer production using Sudan black dye [6].

Substrate Pretreatment: A 10 g of mashed banana peel sample was taken into separate conical flasks, and add 300 ml of 5% NaOH in grain wastewater. The pretreated sample was subjected to the autoclave for 1 hour to break down cellulose from the lignin hold. The pretreated solution was drained off using a muslin cloth. The hydrolysate was subjected toDinitrosalicylicacid method (DNSA) to estimate the reducing sugar content in the substrate [7].

Production and optimisation of biopolymer: The fermentation media (g/L): Glucose: Fructose: Sucrose, 3:2:1, 20; Potassium dihydrogen phosphate, 5; Magnesium sulfate, 0.2; Ammonium sulfate, 1; citric acid, 2; Boric acid, 0.006; Zinc chloride, 0.006; Ferric chloride, 0.0024 and Calcium Carbonate, 0.02) was prepared, pH was adjusted to 7 with phosphate buffer and sterilized. The medium was inoculated with 1% (v/v) of PS1 and PS2 overnight at 180 rpm in a shaking incubator.

To optimise the fermentation process for biopolymer production pH, nitrogen sources, and C/N ratios were varied. The biopolymer production was studied using the production media in pH 5 to 8. The culture was grown for 48 h at room temperature in production media by varying nitrogen sources and C/N ratios from 10:1 to 30:1 at pH 7.0. The biopolymer was extracted using the Sodium hypochlorite extraction method.

FT-IR analysis of biopolymer: The extracted biopolymer was subjected to FT-IR analysis, and it was analysed in a wavenumber range of 4000 – 400 cm^{-1} using an FT-IR spectrometer.

Gas Chromatography mass spectroscopy analysis: The extracted biopolymer from PS1 was carried out on a gas chromatograph-mass spectroscopy analysis (Shimadzu, GCMS QP2010). The extracted biopolymer was subjected to hydrolysis and methylation to identify the monomers according to the protocol described by Rosengart et al. [8].

Preparation of Biopolymer: The synthesised biopolymer was dissolved in 0.1% acetic acid,

and the undissolved particles were removed by centrifugation for 8000 rpm. ThesolutionpHwas neutralised with 1N NaOH to precipitate the biopolymer. The precipitated biopolymer was recovered by filtration, washed extensively with deionised water to remove salts. The biopolymer solution was kept under constant stirring overnight and stored at 4°C [9].

Seedling bioassay: TNAU Maize Hybrid CO 6 were collected from Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore which was used as a seed source for this study. The seeds were surface sterilized in immersing with 10 % of sodium hypochlorite solution for 10 minutes and then washed three times with double distilled water. The surface sterilised seeds subjected for 15 minutes with double distilled water, crab shell extract (CE), green polymer (GP), and neutralized synthesised biopolymer at different concentrations (0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 % w/v). The soaked seeds were placed in a germination sheet with five mL of double-distilled water. Each setup was made in triplicates with 50 seeds and maintained at room temperature. Sealed germination sheets were placed in double distilled water, CE, GP and synthesised biopolymer to maintain the required moisture level. The treated seeds were subjected to a germination test to evaluate the treatment effect on germination rate, seedling length, Seed vigour index (SVI) and biomass. The above parameters were recorded after 14 days of seed sown [10,11].

Measurement of Physiological characteristics of seedlings: The seed germination tests were performed with 50 primed seeds for each treatment, and the experiment was replicated three times in a germination paper (12 cm×18 cm). Then, seeds were stored at room temperature under the alternating cycle of 12-hour light and 12-hour dark for 14 days.

The germination index and mean germination time were calculated as *Germination Index*=Σ(*Gt*/*Tt*) and *Mean Germination Time* =Σ(*Gt*×*Tt*)/Σ*Gt*, respectively, where *Gt* is the number of germinated seeds on Day *t*, *Tt* is the time corresponding to *Gt* in days [10,11].

Measurement of enzyme activity

Alpha (α) amylase activity: The α-amylase activity of maize seeds was estimated at various germination stages of 0, 2, 4, 6, 8, 10, 12 and 14 days of germination. The germinating seeds were subjected to homogenization using 100 mM sodium acetate buffer at 4 °C and maintained the buffer pH 4.7. The enzyme activity of the homogenised solution was recorded at 560 nm [10,11].

Protease assay: The protease activity of maize seeds was estimated at various germination stages of 0, 2, 4, 6, 8, 10, 12 and 14 days of germination. The seeds were homogenised using phosphate buffer (100 mM) at 4 °C. The reaction mixture was subjected to the Lowry method [12,13].

Starch estimation: Quantification of starch was done by performing Anthrone Method for germinating seeds. Two grams of uniformly germinated seeds on different days were homogenized in ethanol (85% w/v) and heat for 10 minutes. The homogenate was strained and centrifuged for5000 rpm for 10 minutes at 4°C. The supernatant was separated and allowed to react with Anthrone reagent and recording the absorbance at 630nm[14].

Protein estimation: Quantification of total proteins was done by performing Lowry's Method for germinating seeds. Two grams of uniformly germinated seeds at different days were homogenised in a pre-chilled five volumes of phosphate buffer (1:5). The homogenate was filtered through Whatman filter paper and centrifuged at 8000 rpm for 10 minutes at 4°C. The supernatant was allowed to react with lowry's reagent and recording the absorbance at 610nm [12,13].

Statistical analysis: The experimental data were performed using ANNOVAthrough means of DOSBox (DOSBox version 0.74). All the

experiment was duplicated, and treatment was performed thrice.

Results and Discussion

Biopolymer Production From mixed Fruit wastes

The biopolymer production using *Enterobacter cloacae* and isolated bacterial strains (PS1and PS2) from banana peel wastes were investigated. The process conditions, including pH, nitrogen sources and C/N ratios, were optimised to obtain the maximum yield.

Effect of pH

The biopolymer production from banana peel wastes using *Enterobacter cloacae* and isolated bacterial strains (PS1and PS2) was investigated at different pHs from 5.0 to 8.0, as represented in Fig.1.

The biopolymer production was significantly increased on varying the initial pHs from 5.0 to 7.0, and it was considerably decreased at above 8.0. It was observed that the low and high pH of the fermentation media inhibited the microorganism activity, which led to low biopolymer production. The maximum output of biopolymer using *Enterobacter cloacae* and isolated bacterial strains (PS1and PS2) at pH 7 was found to be 5.3, 4.9 and 3.4 g/L, respectively. Hence, the optimum pH for biopolymer production was observed to be 7. A similar observation was reported the maximum output of biopolymer from canola oil waste was obtained at pH 7.0 using *Bacillus sp* [15].

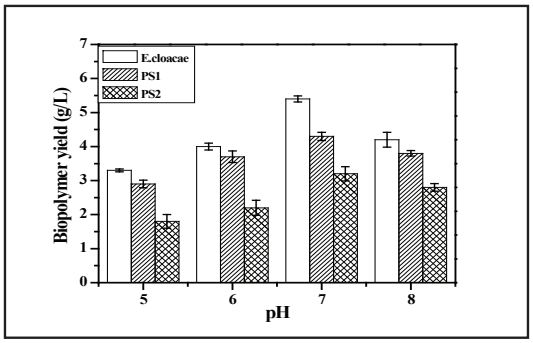

Fig.1. Biopolymer production at different pHs

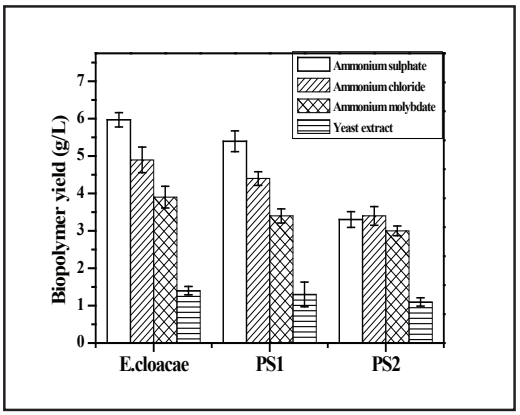

Fig.2. Biopolymer production at different nitrogen sources

Effect of different nitrogen sources:

The nitrogen sources, including ammonium sulphate, ammonium chloride, ammonium molybdate and yeast extract, were individually investigated at 1.0 g/l concentration. As shown in Fig.2, the maximum biopolymer production from banana peel wastes using *Enterobacter cloacae* and isolated bacterial strains (PS1 and PS2) occurred in the presence of ammonium sulphate as a nitrogen source. However, the lowest level of biopolymer production was formed in the yeast extract supplemented fermentation medium. The essential amino acid for microorganism growth may be inhibited at the supplementation of yeast extract. Among the three strains, *Enterobacter cloacae* yielded the maximum biopolymer. The maximum biopolymer yield at 1 g/L of ammonium sulphate for *Enterobacter cloacae,* PS1 and PS2, were 5.9, 4.7 and 3.2 g/L, respectively. Previously, Albuquerque et al. obtained 60% of PHB accumulation from sugar molasses at 1 g/L of ammonium sulphate using *Bacillus megaterium* [16].

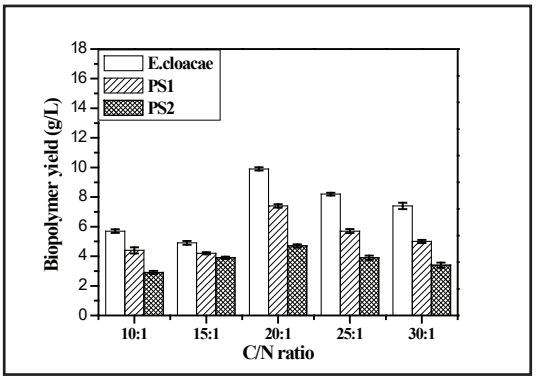

Fig. 3. Effect of C/N ratios on biopolymer yield

Effect of different C/N ratios:

The biopolymer production from mixed fruit wastes at different C/N ratios from 10:1 to 30:1 was investigated at the optimum pH 7.0 using *Enterobacter cloacae* and isolated bacterial strains (PS1 and PS2) as represented in Fig.3.

It was noted the biopolymer production was increased from 6.2 to 8.4 g/L with increasing the C/N ratio from 10:1 to 20:1 for *Enterobacter cloacae.* A similar trend was followed by isolated bacterial strains (PS1 and PS2). At 25:1 and 30:1C/N ratios, the yield of biopolymer was slightly decreased due to substrate inhibition. Enterobacter cloacae have produced a higher yield among the three strains than isolated bacterial strains at pH 7.0. The maximum biopolymer production from mixed fruit wastes was estimated at 20:1 C/N ratio for *Enterobacter cloacae* and isolated bacterial strains (PS1 and PS2). Previously, Albuquerque et al. obtained 74.6% of PHA content from sugar molasses at 20:1 C/N ratio using mixed microbial culture [17].

FT-IR analysis

The FT-IR analysis was carried out to identify different groups present in the produced biopolymer by isolated bacterial strain PS1(Fig. 4). The FT-IR spectrum of biopolymer showed bands at 3429, 2983, 2674, 1727, 1484, 1226, 1183, 900 and 747 cm^{-1}. The intense broad absorbance at 3429 $cm^{-1}$is assigned to the –O–H stretching present in the biopolymer, as reported previously [17]. The characteristic peaks at 2983 and 2674 cm^{-1}correspond to the –CH stretching of the obtained biopolymer. The absorbance peak at 1727 $cm^{-1}$is attributed to the –C**=**O stretching present in the ester carbonyl group of the biopolymer. A $–CH_3$ stretching of the biopolymer occurred at 1484cm^{-1}. The characteristic peaks at 1226 and 1183 cm^{-1} are assigned to the –C–O– stretching of the biopolymer. The strong absorption peaks at 900 and 747 cm^{-1} are designated to the –CH stretching of the biopolymer. Based on the FTIR results, the produced biopolymer corresponds to PHB. A similar observation was reported by Senthilkumar and Prabakaran[18]. They observed that the characteristic groups, including –CH, –C=O, –C–O– and CH_3 were identified in the PHB obtained from diesel using *A. eutrophus*(17*).* The FT-IR (Fig. 5) analysis was carried out to identify the different groups present in the produced biopolymer by isolated bacterial strain PS2. The FT-IR spectrum showed bands at 3434, 2969, 1727,1452, 1377, 1279 and 900 cm^{-1}. As reported previously, the absorbance peak at 3434 $cm^{-1}$is attributed to the –OH stretching present in the biopolymer. The characteristic peak at 2969 cm^{-1} corresponds to the –CH stretching of the biopolymer. The strong absorbance peak at 1727 cm^{-1} is designated to the –C**=**Ostretching present in the ester carbonyl group. The characteristic peaks at 1452 and 1377 cm^{-1} are assigned to the$–CH_3$bending of methyl groups. The absorbance peak at 1279 cm^{-1} is attributed to the –C–O– stretching of the biopolymer. The peak at 900 cm^{-1}corresponds to –CH stretching of the biopolymer. Based on the FTIR results, the obtained biopolymer corresponds to PHB. A similar observation was reported by Senthilkumar and Prabakaran [18]. They observed that the distinct groups, including –CH, –C=O, –C–O– and CH_3 were identified in the PHB from diesel using *A. eutrophus* [18,19].

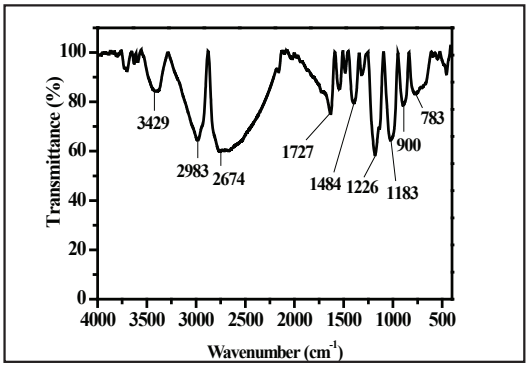

Fig. 4. FT-IR spectrum of biopolymer produced by isolated bacterial strain PS1

GCMS analysis

The result of the GC-MS analysis as shown in fig. 6, the analysis elucidated the characteristic fragmentation patterns, suggesting the presence of PHB. An extracted biopolymer from PS1, which showed major peaks with a retention time of 11.6 and 12.2 minutes, were identical to those of the dimer methyl esters of 3HV and 3HBV, respectively. The identified molecules were compared in the Gas Chromatography database; the major peak symbolizes isopropyl ester of 2-butenoic acid approving the polymer as PHB. The fragmentation patterns were in concordance with the results given by Mohan et al.[19].

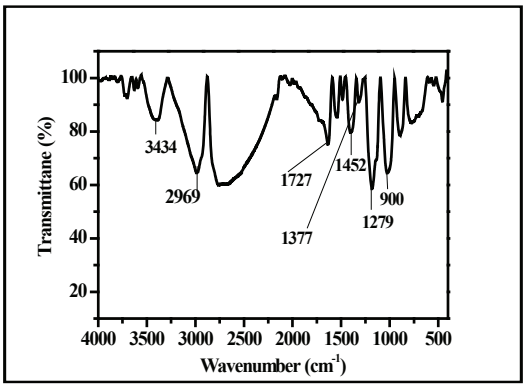

Fig. 5. FT-IR spectrum of biopolymer produced by isolated bacterial strain PS2

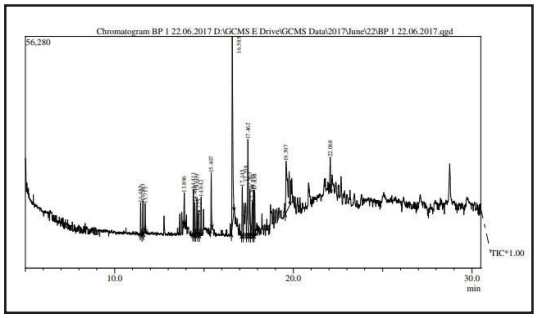

Fig. 6. GC-MS Analysis of biopolymer produced by isolated bacterial strain PS1

Effect of Seed Invigouration treatments

Effect of synthesized biopolymer on starch content in germinating seeds:

The mobilisation pattern of starch was studied during the germination of maize seed. The seed tissues were taken on 0, 2, 4, 6, 10, 12 and 14 days of seedlings for starch estimation. A slow and similar starch mobilization pattern was observed at zeroth day and first day in all treatments. From the third day onwards, the starch started to mobilise rapidly. The maximum mobilisation was observed at 1.6% of BP 1 treated seeds. The biopolymer treated seeds showed higher starch mobilisation when compared to control, CE, and GP treated seeds, as shown in Fig.7.

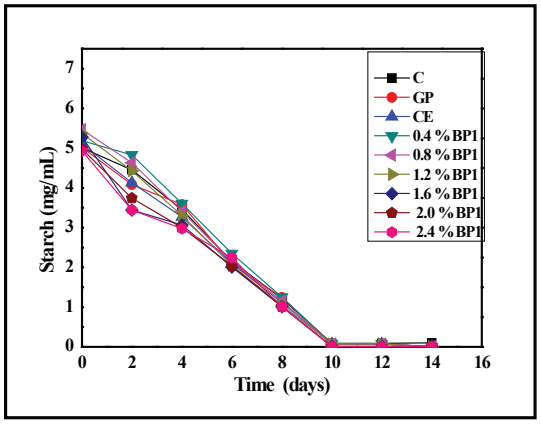

Fig. 7. Effect of Biopolymer on Starch content in Germinating Seeds

After 14 days, the trace level of starch content was observed could in biopolymer treated seeds. At a low concentration of 100 mM NaCl and 25 mM Na_2CO_3, the starch mobilisation was significantly lower than the hydropriming (control). Further, the increase in salt concentration of 300 mM NaCl and 75 mM Na_2CO_3drastically decreased the starch mobilisation. Hence, the starch mobilisation was linearly and negatively correlated with salt stress.

Effect of synthesized biopolymer on protein content in germinating seeds:

The protein mobilisation pattern was estimated in the stage of seed germination. The seed tissues were collected for 14 days (0, 2, 4, 6, 10, 12 and 14 days) to measure the protein content. The study results demonstrate that the protein content was significantly increased from the first day and reached a maximum on the fifth day in all the treatments. After the fifth day, the protein content was sharply declined till the tenth day (Fig.8).

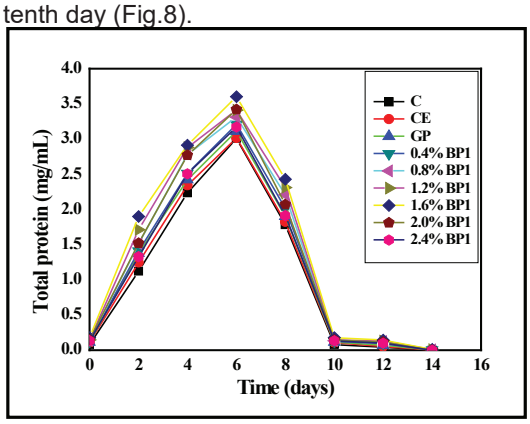

Fig. 8. Effect of Biopolymer on Protein Content in Germinating Seeds

After 14 days, the trace level of protein content was observed in biopolymer-treated seeds. At a low concentration of 100 mM NaCl and 25 mM Na_2CO_3, the protein mobilization was considerably lower than the control. Moreover, the increase in salt concentration of 300 mM NaCl and 75 mM Na_2CO_3 significantly decreased the protein mobilization. Hence, the protein mobilization was linearly and negatively correlated with salt stress.

Effect of synthesized biopolymer on α-amylase in germinating seeds:

The effect of the synthesised biopolymer on the starch and protein mobilisation in correlation with enzyme activity was studied. The α-amylase activity was measured for 14 days (0, 2, 4, 6, 10, 12 and 14 days)in germinating seeds, as shown in Fig.9.

On the day of germination, the negligible activity of amylase was observed in all treatments. The amylase activity was increased from the first day to the sixth day and declined rapidly on the tenth day at all treatments. After the tenth day, the trace level of amylase activity was noticed in biopolymer-treated seeds. On the fifth day, amylase activity was found to be maximum at 1.6% of BP1 treated seeds. The α-amylase activity was slightly decreased at 2.0 and 2.4% of BP1 treatment. The α-amylase activity of maize seeds was significantly decreased under NaCl (100, 200 and 300 mM), and Na_2CO_3 (25, 50 and 75 mM) stress conditions. Several authors reported that the decrease of enzyme activity might be due to higher accretion of Na^+ ions that interfere with potassium uptake causing severe water loss and necrosis in maize [20,21].

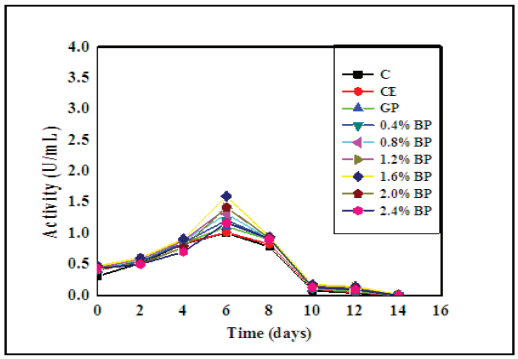

Fig. 9. Effect of Biopolymer on α-Amylase in Germinating Seeds

Effect of synthesised biopolymer on protease in germinating seeds:

The effect of the synthesised biopolymer was to correlate the mobilisation of protein with protease activity. Protease activity was measured for 14 days (0, 2, 4, 6, 10, 12 and 14 days) in germinating seeds, as represented in Fig.10.

On the zeroth day, the negligible activity of protease was observed in all treatments. The activity was increased from the first day to the sixth day and decreased drastically on the tenth day in all treatments. The trace levels of protease activity were noted on the 10th day. On the sixth day, the protease activity was high at 1.6% of BP1 treatment. The protease activity was slightly decreased at 2.0 and 2.4% of BP1 treatment. The protease activity was also affected by different salt stress conditions like amylase activity [22].

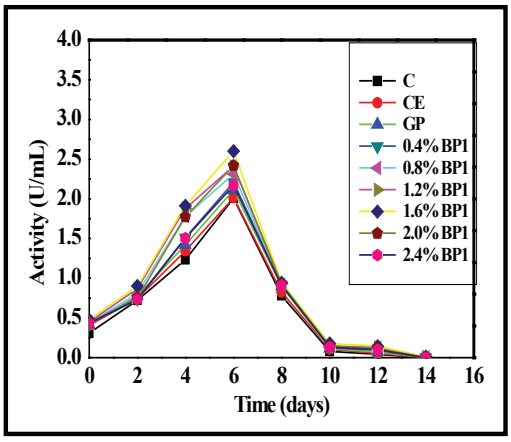

Fig. 10. Effect of Biopolymer on Protease in Germinating Seeds

Conclusions

Enterobacter cloacae and isolated bacterial strains were found to be capable of producing biopolymer using the synthetic medium [23]. The study results reveal that the biopolymer yield using *Enterobacter cloacae,* PS1and PS2 from mixed fruit wastes as a sole carbon source was obtained to be 8.4, 6.2 and 3.9 g/L, respectively at pH 7.0 and C/N ratio 20:1 [24]. *Enterobacter cloacae*'s biopolymer synthesising capacity was more compared to the other two bacterial strains (PS1 and PS2). Among the isolated bacterial species (PS1 and PS2), the bacterial strain PS2 yielded low biopolymer when compared to other strains. Based on the FTIR results, the obtained biopolymer using *Enterobacter cloacae* was similar to chitosan. Moreover, the synthesised biopolymer using PS1 and PS2 was obtained to be identical to PHB (Polyhydroxybutyrate).

The synthesised biopolymer (BP 1) (0.4 to 1.6 %) showed a higher seed vigor index, seedling rate and germination percentage, increased enzymatic activity in germinating seeds when compared to control, CE and GP treatments. The BP 1 treatment at 2.0 and 2.4 % caused a slight decline in seedling growth and enzymatic activity in germinating seeds, thereby inhibiting seedling growth. The results also indicated that seedling length, germination percentage, biomass, and relative water content was decreased with increasing salt concentration. The growth inhibitory effect of NaCl and Na_2CO_3 was more pronounced in 300 mM and 75 mM compared to control. Therefore, we conclude that 1.6 % of BP 1 treated seeds helped enhance seedling growth by up-regulating the responsible enzymes to mobilise starch and protein.

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Conflict of interest

The authors have no conflict of interest.

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