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Original Research Article

Use of Banana Waste for the Production of Cellulolytic Enzymes under Solid Substrate Fermentation Using Bacterial Consortium

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A B S T R A C T

The present study aims to the use of the bacterial consortia for the degradation of the banana waste by solid state fermentation for cellulase production. Four bacterial strains were tested to find their ability to produce cellulases, which catalyze the degradation of cellulose, which is a linear polymer made of glucose subunits linked by β -1, 4 glycosidic bonds. This all four bacterial strain was noticed to show maximum zone of hydrolysis of carboxy-methyl cellulose and four bacterial mixture (consotia) produce higher activities of the cellulases were determined by Filter paper assay (FPA), Carboxy-methly cellulase assay (CMCase) and β -D-glucosidase assay respectively. The production patterns of these enzymes were studied during the growth on the organisms for a period of 40 days. Bacterial consortia exhibited high level of enzyme activities and pattern of production. Maximum specific activities of enzymes were obtained between 15 to 25 days of culture growth.

Keywords

Banana waste; Bacterial consortia; Solid substrate fermentation.

Introduction

Banana is one the most extensively consumed fruits in the world and India are one of the largest producing countries of this fruit, which is cultivated in 4.796 $\times 10^5$ ha yielding 16.37 $\times$ 10^6 t of banana (ICME, 2005). Main horticulture crops in Gujarat state of Anand district are Banana, Citrus, Mango, Papaya, Aonla and Sapota (WAPCOS, 2008). Area wise Banana is grown over an area of 11693 ha, followed by Citrus and Mango with an area of 4292 ha and 1919 ha respectively and Papaya, Aonla and Sapota being grown over an

cellulose and lignin. Most of the residual waste produced due to banana cultivation is discarded by farmers into nearby rivers, lakes and on roads, which causes a serious environmental concern (Pandey A *et al*., 1999, Brand D *et al*., 2002). In recent year's use of this agro industrial waste as substrate in bioprocess for production of a value added products. Solid state fermentation (SSF) has important industrial application including manufacture of selected high value microbial products (Krishna and Chandrasekharan, 1996). The main

Fig.1 Horticulture cropping patterns in Anand districts

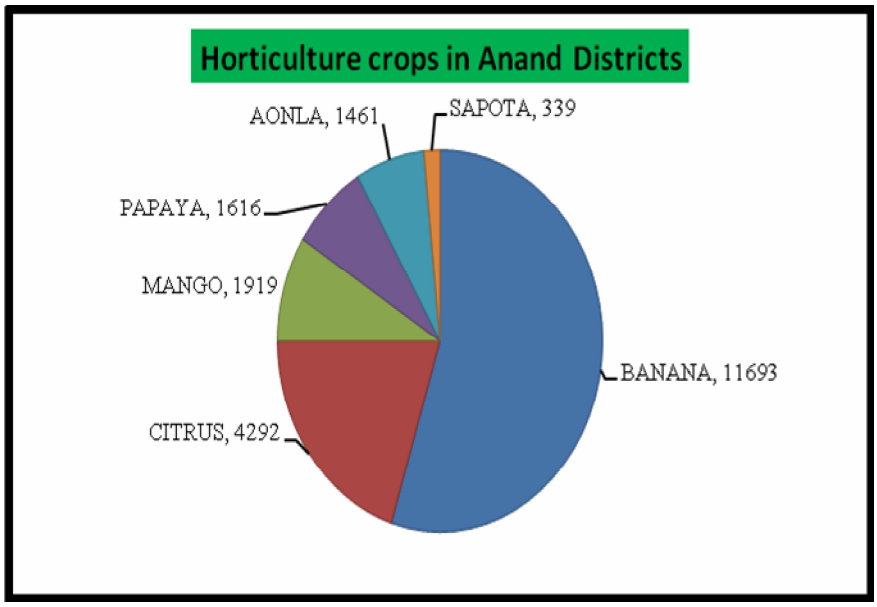
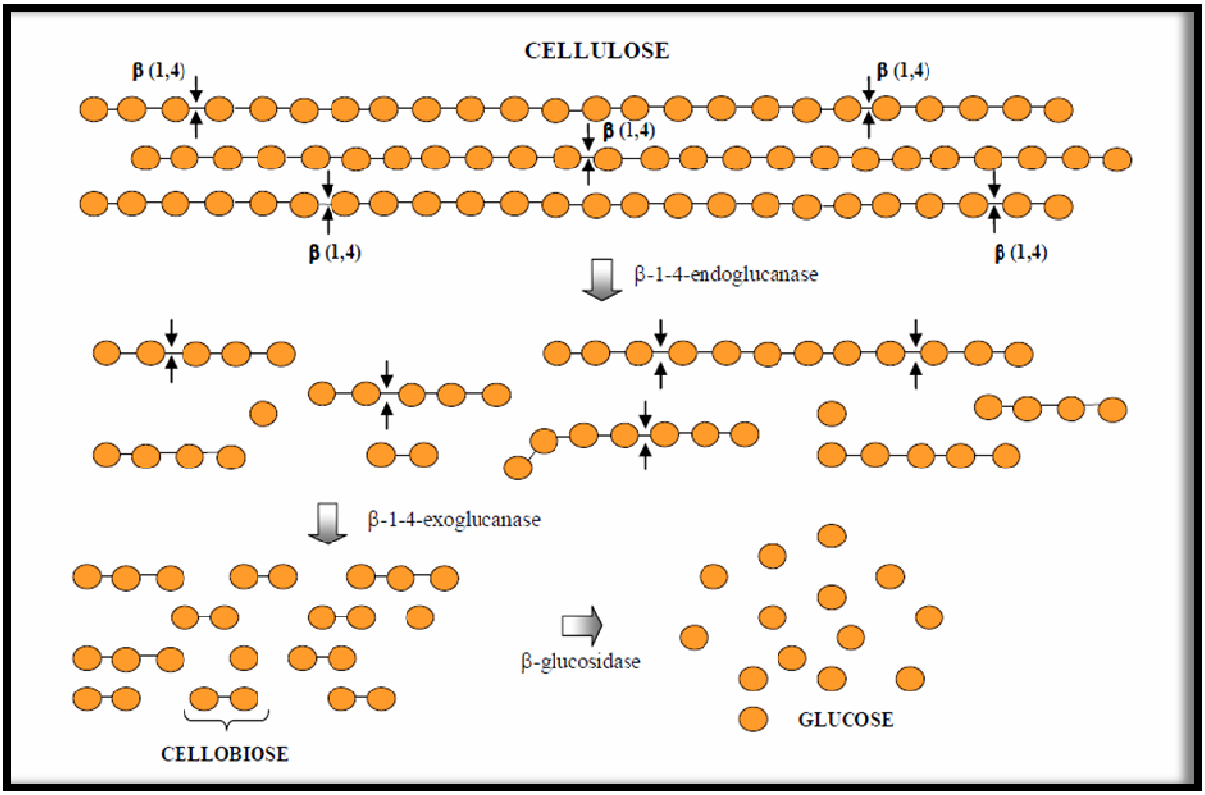


Fig.2 Schematic representations of the cellulase enzymes over the cellulose structure



residual wastes of the banana crops are leaves and pseudostem (flattened and modified stem). This 'pseudostem' consists of concentric layers of leaf sheath and column of large leaves, both containing high level of lignocellulose (Reddy.G.V. 2001). These lignocellulosic materials are efficient substrates for some bacteria, which produce cellulolytic enzymes that have numerous applications in industrial processes for food and feed, textile, laundry and detergent use.

Cellulose is a high molecular weight linear homopolymer of repeated units of cellobiose (two anhydrous glucose rings joined via a β-1, 4 glycosidic linkages (Klemm *et al*, 1988).

Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. The complete degradation of cellulose is made by a cellulolytic enzyme system. It has been established that there are three main types of enzymes found in the cellulase system that can degrade cellulose: exo-β-1, 4-glucanase, endo-β-1, 4-glucanase and β-glucosidase. The endoglucanases act internally on the chain of cellulose cleaving β-linked bond liberating non reducing ends, and exoglucanase remove cellobiose from this non-reducing end of cellulose chain. Finally, β-D-glucosidase completes the saccharification by splitting cellobiose and small cello oligosaccharides to glucose molecule [Sun and chang.2002, Prasad *et al*., 2007, Cao and tan, 2002]. A schematic representation of the cellulase enzymes over the cellulose structure is shown in Fig. 2. A wide variety of cellulolytic fungi and bacteria have been reported.

In the present study use of the bacterial consortia including *cellulomonas cartae*, *pseudomonas fleuroscence*, *Pseudomonas putida*, *Bacillus megaterium* were assessed for their ability to produce cellulolytic enzymes such as endo-1,4-β-D-glucanase (carboxy methyl cellulase, CMCase EC 3.2.1.4) and exo-1,4-β-D-glucanase (FP activity EC 3.2.1.91) and β- glucosidase by solid substrate fermentation on banana residual waste. The dynamics of these extracellular enzymes were studied during the growth of these organisms on banana pseudostem waste.

Materials and Methods

Primary screening for cellulolytic activity by plate assay

The presence of endoglucanases was tested using the media proposed by Hart *et al* (2002). The individual bacteria grown on basal salt media supplemented with 1% carboxy methylcellulose used for carbon source. The pure cultures were inoculated in the centre with almost equal amounts and incubated at 37^0c until substantial growth was recorded. The petri plates were flooded with Congo red solution (0.1%), and after 5 min the Congo red solution was discarded, and the plates were washed with 1M NaCl solution allowed to stand for 15– 20 minutes. The clear zone was observed around the colony when the enzyme had utilized the cellulose.

Preparation of Bacterial Consortia

The main aim of this study is to develop some successful bacterial consortium that can concomitantly degradation different components of the banana waste with the help of their enzymes in less span of time under natural conditions without producing any foul odour.

Effective bacterial consortia for the degradation of banana waste were prepared as per method proposed by(Rahman, 2002).To prepare successful microbial consortium, bacterial cultures must be compatible with each other in order to concomitantly produce all these enzymes required for the degradation of banana wastes. Consortia were prepared and incubated overnight at 37^{o}C in 120 rpm. The compatibility of the bacterial strains within the consortia was checked by cross streaking in N agar plate. Microbial consortium was prepared by inoculating over night grown four bacterial strains in 20 ml of nutrient broth.

SSF substrate preparation, inoculation and culture conditions

Agricultural wastes of banana plants were collected, dried and divided into pseudostem and the leaf portion, each of which were cut into ~2 cm pieces. Twenty-five grams of each portion was placed in 500 ml conical flasks and moistened with 75 ml of distilled water. The flasks were autoclaved for 2 h at 121 °C and they were inoculated separately with 20 ml of individual bacterial strain and consortia. Cultures were incubated at 32±2°C in a BOD incubator and samples were collected at every 5 day interval until the 40th day.

Sampling, extraction and analytical methods

Enzymes were extracted from 5 g of sample with 20 ml of cold 0.05 M acetate buffer (pH 6.5). The homogenate was filtered through nylon cloth of 200 meshes and the filtrate was centrifuged at 6000 **x** *g* at 4 °C for 20min. The supernatant was analyzed for activities of carboxy methyl cellulase (CMCase), filter paper activity (FPase) and β - glucosidase activity.

Analytical methods

Filter paper assay (FPA)

Filter paper activity of the culture filtrates was determined according to the method of Mendel's and Weber (1969). The activity of filter paper cellulase was assayed using whatman No 1 filter paper (natural crystalline cellulose) as substrate. Whatman filter paper strips containing 50 mg weight was suspended in 1 ml of 0.05 M sodium citrate buffer (pH 4.8) at 50 °C in a water bath. Suitable aliquots of enzyme source were added to the above mixture and incubated for 60 minutes at 50°C.

After incubation, the liberated reducing sugar was estimated by the addition of 3, 5-dinitrosalicylic acid (DNS). After cooling, the colour developed in tubes was read at 540 nm by using the spectrophotometer. Appropriate control without enzyme was simultaneously run. Activity of cellulases was expressed in filter paper units.

One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 μ mole of reducing sugar from filter paper /ml /h.

Carboxy methyl cellulase enzyme assay (CMCase)

Activity of endoglucanases in the culture filtrates was quantified by carboxy-methyl cellulase method (Ghosh 1987). The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50 °C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture fi ltrate with appropriate dilution was added to the reaction mixture and incubated at 50 °C in water bath for one h. appropriate control without enzyme was simultaneously run.

Estimation of reducing sugar by DNSA method

The reducing sugar produced in the reaction mixture was determined by Dinitro- salicylic acid (DNS) method (Miller 1959). 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the colour developed was read at wavelength 540 nm by using the spectrophotometer.

One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar /ml /h.

Calculation

Enzyme activity (U/ml) for CMCase activity and FPase activity =

O.D at 540nm ×µM of glucose (from standard graph) ×dilution factor
0.1 ×Incubation time (min) ×aliquots of enzyme (ml) ×MW of glucose (gm)

β **- D-Glucosidase assay**

Activity of *β* - glucosidase in the culture filtrates was quantified based on the method of Herr (1979). For the determination of *β* - D-glucosidase activity the assay mixture contained 0.2 ml of 5mM p-nitro phenyl *β* - D-glucopyranoside (PNGP) in 0.05 M citrate buffer pH 4.8 and 0.2 ml of diluted enzyme solution with appropriate controls. After incubation for 30 min at 50 °C, the reaction was stopped by adding 4 ml of 0.05 M NaOH-Glycine buffer (pH 10.6) and the yellow colour *p* - nitro phenol liberated was determined at 420 nm by using the spectrophotometer.

One unit of *β* – glucosidase activity was defined as the amount of enzyme liberating 1 *µ* mole of *þ* – nitrophenol /ml /h under standard assay conditions.

Results and Discussion

Plate Assay

The bacterial cultures *Cellulomonas cartae, Pseudomonas fluorescence, Pseudomonas putida, Bacillus megaterium* were screened for their cellulolytic activity. All the bacterial cultures produced zones of hydrolysis in CMC agar plates within 3 days and results were represented (fig.3, table.1).
Among the four bacterial strain, *Bacillus megaterium* was detected to produce maximum zone of hydrolysis on (4.6 cm) of carboxy- methyl cellulose agar plate, whereas *Cellulomonas cartae, Pseudomonas fleuroscence, Pseudomonas putida* produced zone diameters of 4.2,3.2 and 2.9 cm respectively

Preparation of bacterial consortia

For the preparation of the consortium, colonies were plated on minimal agar medium supplemented with carboxy methyl cellulose and incubated overnight at 37 ºC. After 12 hours the strains, at log phase, were inoculated onto 250 mL shake flasks containing 100 ml nutrient broth and incubated at 37 ºC at 200 rpm for 12 hours.

A sample of 5 mL from each broth was added to a sterile falcon tube and centrifuged at 5000 rpm for 15 minutes. The supernatant was discarded and normal saline solution was added to the pellets and vortexes well. A volume of 0.1 ml of each colony was added to nutrient broth and the broth was incubated overnight. The consortium in nutrient broth was used to inoculate flasks for kinetic study and biodegradability test.

Figure.3 Screening of cellulolytic bacterial cultures on CMC agar plate

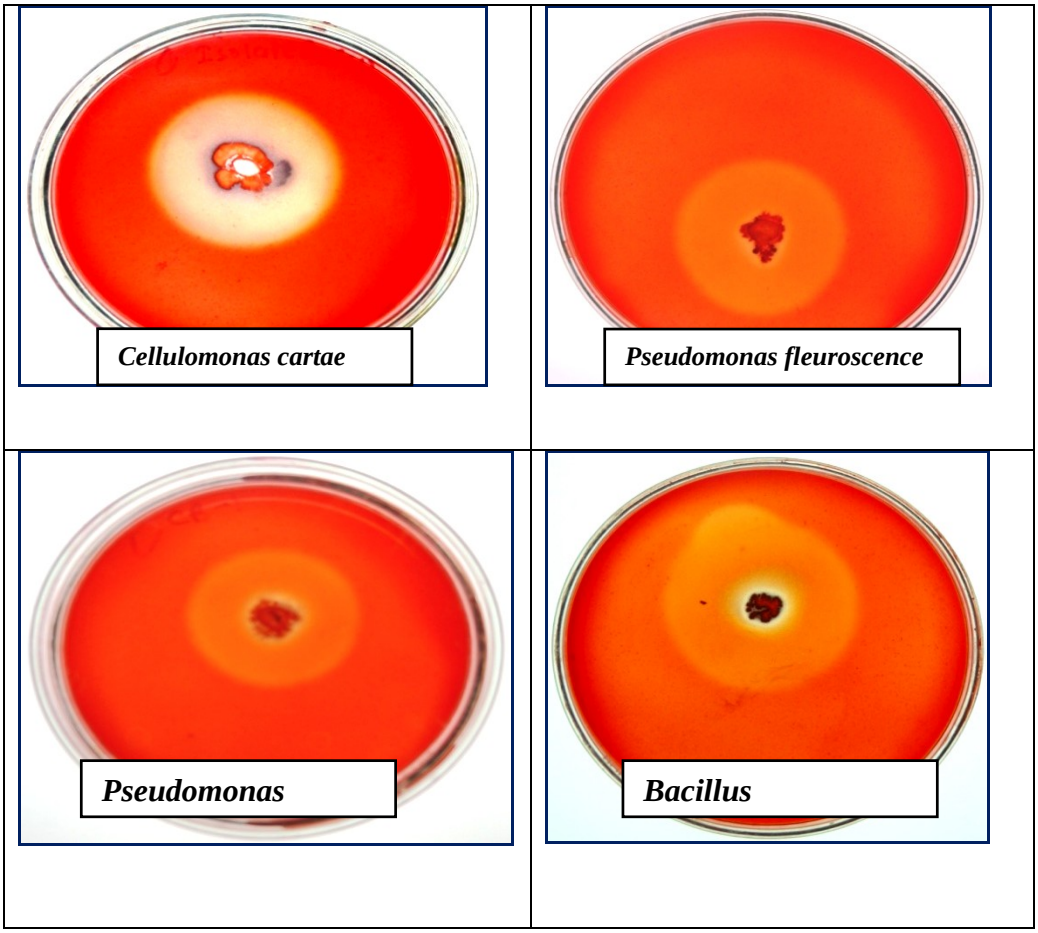


Table.1 Biometric features of cellulolytic bacterial cultures

Sr.No.	Bacterial culture	Colony colour	Colony diameter on CMC agar plate (cm)	Zone of hydrolysis on CMC agar plate (cm)
1	*Cellulomonas cartae*	Bluish white	2.8	4.2
2	*Pseudomonas fleuroscence*	Light green	1.1	3.2
3	*Pseudomonas putida*	Brown	1.2	2.9
4	*Bacillus megaterium*	Pale Yellow	2.5	4.6

Fig.4 Production pattern of exo-1, 4-β-D-glucanase (FP activity) on banana waste

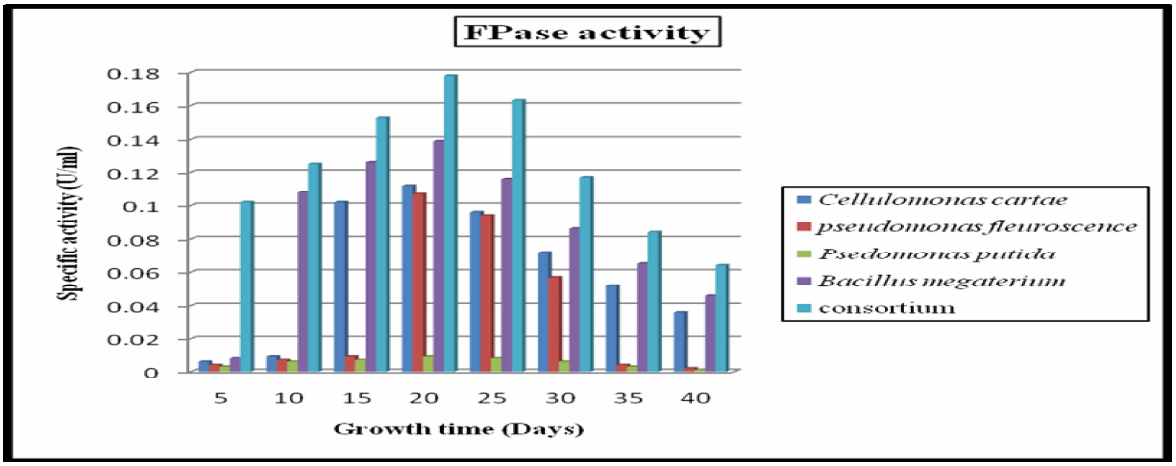


Fig.6 Production pattern of endo-1, 4-β-D-glucanase (CMCase) on banana waste

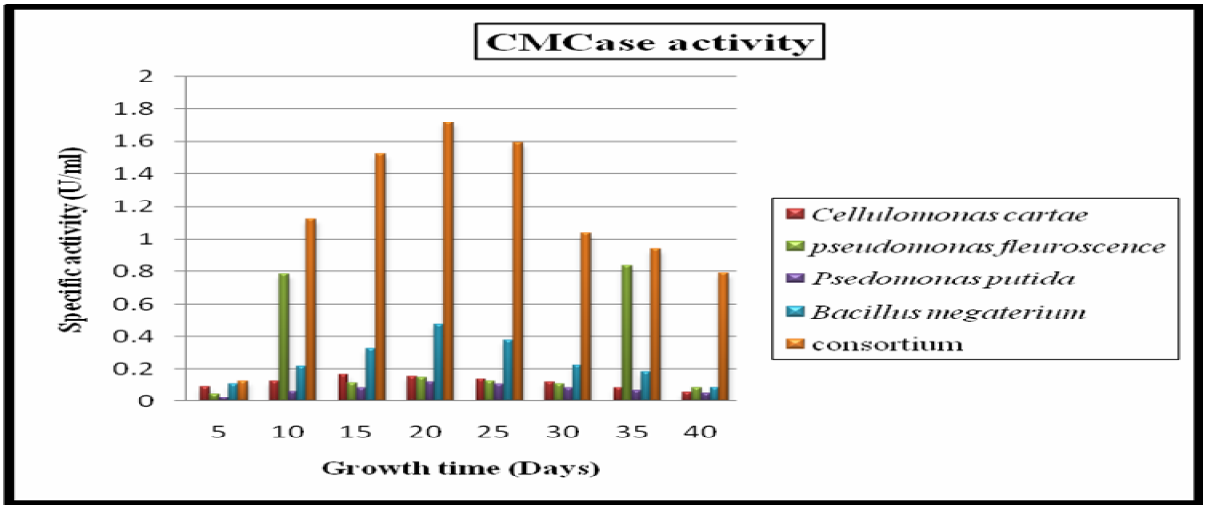
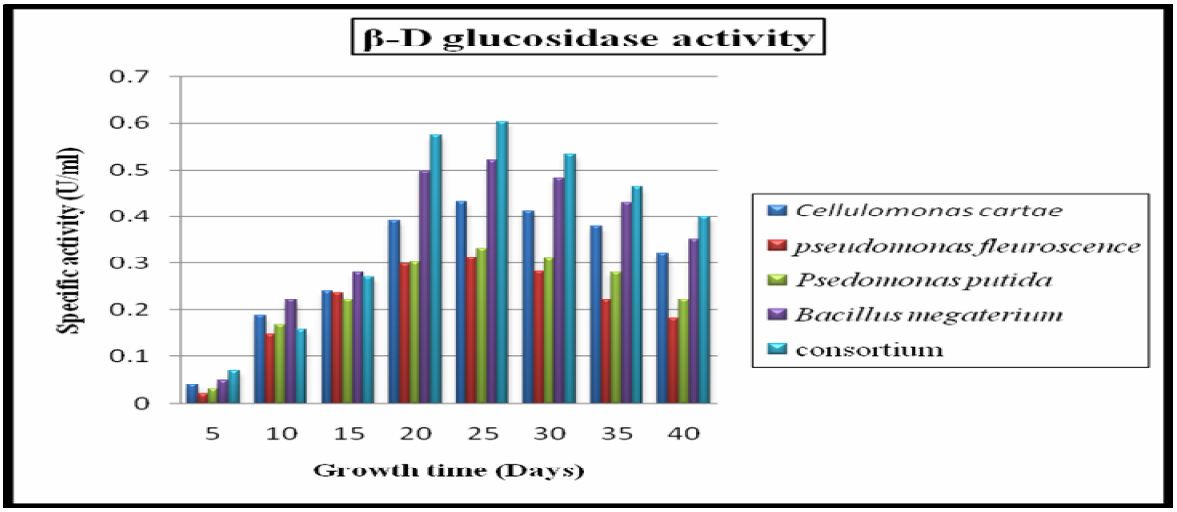


Fig.7 Production pattern of β - D-Glucosidase activity on banana waste



Production pattern of cellulolytic enzymes on pseudostem biomass on banana waste by individual bacteria and bacterial consortia are shown in figure. 5,6,7.

Filter paper assay

Maximal specific activities for all the bacterial strain were observed on the 20th day, afterwards up to the 40th day a gradual decrease is observed. The highest specific activity of filter paper was observed on the day 20th day of the bacterial consortia by giving 0.178 units/ml followed by gradual decrease.

Endo-1, 4-β-D-glucanase (carboxy methyl cellulase, CMCase)

Maximal specific activities for all the bacterial strain were observed on the 20th day, afterwards up to the 40th day a gradual decrease is observed. The highest specific activity of CMCase was observed on the day 20th day of the bacterial consortia by giving 1.716 units/ml followed by gradual decrease.

β **- D-Glucosidase activity**

The specific activity of the extracellular enzymes produced by four individual bacteria and consortium during the degradation of banana pseudostem biomass is presented in Fig. 7. The highest specific activity of *β* - D-Glucosidase was observed on day 25 of the consortia 0.602 units/ml followed by gradual decrease.

Cellulose degradation occurs naturally in complex microbial communities that include many non-cellulolytic organisms. It is therefore likely that the bacterial consortium helps degrade banana crop residue as part of a larger free-living consortium found in banana field soil. This preliminary study provides evidence that the consortium may work in accelerating the decomposition of banana crop residue.

Banana crop cultivation has been on rise at the global level, consequently generating a huge amount of rich residual wastes. There is enormous potential for exploitation of this substrate. The present investigation indicates that microbial bio transformed banana substrate can be a rich source of organics. These organics are produced by the dynamic kinetics of the enzymes, which are produced by cellulolytic bacterial strains.

In the present study, it could be concluded that the bacterial cultures degrade the banana waste of leaf and pseudo stem biomass possess cellulolytic activity. Among these all bacterial cultures, was noticed to show maximum zone of hydrolysis of carboxy - methyl cellulose. It also produced high titers of Filter paper assay (FPase) (0.178 U/ml) Carboxy-methyl cellulase assay (1.716 U/ml) *β*–D-glucosidase (0.602 U/ml). The bacterial consortia in the present investigation need to be further studied in depth for their cellulolytic potential for conversion of cellulosic waste material into useful products.

The present study reveals that banana waste can be used as an alternative substrate to other agricultural/agro-industrial waste, wheat bran/straw, sawdust and bagasse, which are already in use for the production of lignolytic and cellulolytic enzyme production. The SSF condition reveals a cheaper protocol for the production of these extracellular enzymes. The degradation of organic wastes by the bacterial consortia is highly significant. It reduces the time span of degradation and produces no foul odour.

The use of microbial consortium generated through natural selection or improvement of the performance of these microorganisms in organic banana waste degradation through genetic manipulation, may be the best option for the efficient treatment of organic banana waste in the near future.

Further study is needed to extend this work for possible future application of this technology. Future study should be directed at the use of consortium in various forms such as active cultures, lyophilized bacteria and liquid cultures. Future field studies should be conducted using these various microbial forms with the goal of optimizing shelf life and ease of application for farmers. Also, a long-term study should be done to examine what effect herbicide and fertilizer applications may have on performance of the microbial consortia.

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