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EXTRACELLULAR ENZYME PRODUCTION BY ENDOPHYTIC ACTINOMYCETES FROM *SYZYGIUM CUMINI*

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ABSTRACT

Search of new microorganism for enzyme production are increasing around the world. Actinomycetes constitute a potential source of biotechnologically interesting substances, which make it one of the most investigated group. Due to formation of stable mycelia, streptomycetes have ability to secrete an array of different extracellular enzymes including cellulose, amylase, pectinase and chitinase. The purpose of this experiment was qualitative and quantitative study of extracellular enzyme production by endophytic actinomycetes from *Syzygium cumini*. Out of all screened actinomycetes isolates, two streptomycetes isolates were selected for further cellulose and pectinase production and quantification. Production of cellulase, amylase and pectinase by SA 1 and SA 2 isolates and production of chitinase by SA 3 respectively was observed after incubation of 24 hour. Isolates were inoculated in minimal salt solution amended with 1 % (w/v) carboxymethyl cellulose (CMC) for cellulose production, for amylase production Starch Hydrolysis Media, 1 % (w/v) pectin for pectinase production and Basal salt medium for chitinase production. Enzyme activity was assayed as the amount of glucose released in µM/ml/min using dinitrosalicylic acid assay method. The crude cellulose enzyme had maximum activity (0.049 µM/ml/min) after 264 hour of incubation at 35° C while crude pectinase had maximum activity (0.091 µM/ml/min) after 72 hour of incubation at 35° C, whereas crude amylase had maximum activity (0.131 µM/ml/min) after 96 hour of incubation at 35° C, whereas isolate SA 3 shows maximum activity of chitinase (0.042 µM/ml/min) after 120 hour.

Keywords: Extracellular enzymes, Endophytic actinomycetes, Qualitative and Quantitative, Streptomyces

INTRODUCTION

Actinomycetes were also known as actinobacteria, derived from the Greek word where aktis refers to "lightning" and mykes refers to the fungus which was originally classified as an interposed group between fungi and bacteria. Waheeda and Shyam, (2017) reported that endophytic microorganisms are those which do not cause any immediate and negative effect while colonizing living internal tissue. Actinomycetes are considered as a unique group of microorganism which locus between the true bacteria and the true fungi. Nearly all plant species serves as a source for endophytes and accepted as a promising source of novel medicinal compounds (Jasmine and Agastian, 2013). Endophytes are microorganisms that inhabit the internal tissues of plants without causing any negative effects, and actinomycetes isolated from plants have been widely studied due their ability to produce active metabolites. Using microorganism 23,000 of active secondary metabolites was produced, in that 10,000 were isolated from actinomycetes. Of these, Streptomyces species produces 7,600 bioactive compounds. Depending upon the source of isolation the biological function of actinomycetes varies (Janaki, 2017). As like plants, endophytic microorganism has the capacity to produce secondary metabolites thereby it serves as an optimistic source for the novel compound (Machavariani *et al*., 2014). Endophytic actinomycetes are unicellular, Gram-positive aerobic bacteria belonging to class of branched microorganism which are actively involved in the generation of vital compounds by degrading organic matter (Nimnoi *et al*., 2010). In the recent years, endophytic actinomycetes have emerged as an untapped resource of promising drug discovery (Matsumoto and Takahashi, 2017).

Enzymes are the most important products for human needs in the area of industrial, environmental and food technology through microbial sources (Salahuddin *et al*., 2011). Streptomycetes are considered as one of the most important bacteria, due to their ability to develop the soil properties as well as producing several extracellular substances (enzymes) as secondary products.

Solid state fermentation (SSF), a process that occurs in the absence or near absence of water, has been used for the production of various high value added products such as enzymes and other organic components. The substrate acts mainly as source of nutrients for the microorganisms responsible for the fermentation. There are various groups of

microorganisms used in SSF depending on the final product considered necessary to be obtained from the fermentation. The SSF process has been extensively used for the production of high value added products such as enzymes, biofuel, biosurfactants and biopesticides (Singhania *et al*., 2009).

MATERIALS AND METHODS

Collection of the plant material

The *Syzygium cumini* plant samples were collected from their natural habitats. Disease free parts of the plants were cut with the help of a sterile scalpel and placed in plastic bags to store the material.

Isolation of endophytes

Surface sterilization of the plant material:

The collected plant material used for the isolation was first surface sterilized following the method of Dos Santos *et al*., (2003) with few modifications. Plant material was first cleaned by washing several times under running tap water and then cut into small segments. Surface sterilization was performed by sequentially rinsing the plant material with 70% ethanol (C_2H_5OH) for 1 min., then with 0.5% sodium hypochlorite (NaOCl) for 2-3 minutes and finally with sterile distilled water for 2-3 times, then followed by washing with 70% ethanol for 5 seconds. The plant materials were then rinsed in sterile distilled water. Plant material was then dried in between the folds of sterile filter papers.

Isolation of the entophytes:

After proper drying, the surface sterilized plant material i.e. roots were cut into smaller pieces and each piece was placed on potato dextrose agar (PDA) medium supplemented with streptomycin sulphate (250 mg/L). Four explants were put on each PDA plate. All the plates were incubated at 28^o C to promote the growth of endophytes and were regularly monitored for any microbial growth. On observing the microbial growth, sub culturing was done. Each endophytic culture was checked for purity and transferred to freshly prepared PDA plate.

Preservation and maintenance of isolated fungal endophytes:

Isolates were preserved using following methods -

In paraffin mineral oil, stored at room temperature. On agar slant stored in 15% glycerol at 20° C.

Biochemical Assay:

Three endophytic actinomycetes were selected for this study. One isolate (SA 1) was selected for quantification of cellulase and amylase production, second isolate (SA 2) was selected for quantification of pectinase production. Third isolate (SA 3) was selected for chitinase production. These isolates were isolated from root of *Syzygium cumini* plant and preserved in laboratory.

Isolation and preservation of endophytic actinomycetes from plant tissues

The isolation and preservation were carried out by employing Starch Casein Potassium nitrate (SCP) agar media

The composition of the media employed is given below:

Starch Casein Potassium Nitrate Agar (Kuster and Williams, 1964)

Media composition: Starch 10 g, Casein 0.3 g, KNO_3 2 g, K_2HPO_4 2 g, NaCl 2 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.01 g, Distilled water 1litre, Agar 18 g, pH 7.4

The growth media were incorporated with the antibacterial (Rose Bengal 0.035g/L) and antifungal agent 50µg/ml Nystatin (William and Davies, 1965)

Screening for cellulolytic activity (CMC Media):

Media composition: $FeSO_4.7H_2O$ 0.5g, $FeSO_4$ 0.01g, $NaNO_3$ 2.0 g, CMC 10 g, K_2HPO_4 0.05g, $CaCl_2$ 0. 02g, $MnSO_4$ 0.02 g, Agar 18.0 g, Distilled water 1 litre, pH 7.0

Cellulolytic activity was observed by Reese's mineral media containing cellulose (1%w/v) as carbon source. The isolated (SA 1) culture was streaked on alkaline CMC agar plates and checked for clearance due to cellulase production by Congo red method (Wood *et al*., 1988). Congo red was prepared by dissolving 0.5% in double distilled water.

The above plates after full growth of cultures were submerged with 0.1% Congo red dye solution and incubated for 15 min. The Congo red dye was drained off and washed twice with 10mM NaCl solution. The formation of halo zone around the colonies was observed. The presence of halo zones represents the hydrolysis of cellulose. The diameter of the halo zones was recorded.

Screening for Amylase activity:

Starch Hydrolysis Media

Components: Peptone 5 g, Beef Extract 3 g, Starch soluble 20 g, Agar 18 g, Distilled water 1 litre, pH 7.0

Amylase activity was observed on Reese's mineral media containing starch (1%w/v) as carbon source. Prepare starch agar plates and label the plates with organisms. Using a sterile technique make a spot inoculation of test organism (SA 1) agar surface. Incubate the plates for 24 to 48 hr at 37°C. The starch agar plate culture was flooded with gram′s iodine solution and allowed the iodine to remain in contact with medium for 30 seconds. Excess iodine solution after stained the plates was drained off. The development of blue black colour surrounding the growth of test organism was examined. The blue colour of the medium indicates the absence of starch hydrolysis. Clear zone around the culture indicated starch hydrolysis.

Screening of Pectinase activity:

Components: $(NH_4)_2SO_4$ 2 g, K_2HPO_4 2 g, $KHPO_4$ 2 g, Yeast extract 3 g, Pectin 5 g, Agar 18 g, Distilled water 1litre

Isolate SA 2 was streaked on the Pectin agar plate and incubate at 30°C for 3 to 5 days. It was drained with 1% CTAB solution for 30 minutes. Excess CTAB solution will be drained off. Clear zone around Actinomycetes colony shows positive activity.

CHITINASE ACTIVITY (Basal salt medium):-

Media composition: K_2HPO_4 0.21g, KH_2PO_4 0.09g, $MgSO_4$ 0.15g, $FeSO_4$ 0.003g, $MnSO_4$ 0.003g, $ZnSO_4$ 0.003g, $(NH_4)_2SO_4$ 0.075 g, Yeast extracts 0.3g, Distilled water 300ml, Colloidal chitin 3g, Agar 4.5 g

Preparation of colloidal chitin:

10 g. of chitin was dissolved in conc. HCl and kept on shaker for 30 min. 200ml ethanol was added and again kept on shaker for overnight. Next day one liter of distilled water was added and centrifuged at 13000 rpm for 20 minutes at 4°C temperature colloidal chitin in the form of precipitate was repeatedly washed with distilled water until pH reached up to 7.

Screening of chitinase activity:

Isolate SA 3 was streaked on the chitin agar plate and incubate at 30°C for 3 to 5 days. Clear zone around actinomycetes colony shows positive activity.

Quantification of chitinase activity:

Colloidal chitin broth medium was prepared and isolate was inoculated in broth and incubated at 37° C. After every 24 hour 15 ml of medium was withdrawn in aseptic condition. it was centrifuged and supernatant was used as crude enzyme extract.

Substrate preparation for SSF

Five grams of dry wheat bran having both coarse and fine particles in 1:1 ratio (w/w) contained in a 250 ml Erlenmeyer flask and was supplemented with 2ml salt solution containing 0.5% NH_4NO_3, 0.2% KH_2PO_4, 0.1% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$. The initial moisture level in the substrate was adjusted by adding adequate amount of distilled water. The thoroughly mixed substrate was autoclaved for 20 min at 121° C (15 lb) and cooled to room temperature before inoculation.

Solid-state fermentation

Under aseptic conditions, the sterilized solid substrate medium was inoculated with 1ml actinomycetes spore inoculum, the spore suspension contained 2×10^7 spores/ml. The contents were mixed thoroughly and the flasks were placed in an incubator at 30° C for desired time intervals. All the sets were prepared in duplicate.

Enzyme extraction

To the fermented substrate an adequate amount of distilled water containing 0.1% Tween 80 was added to get a total extraction volume of 100 ml. The contents were thoroughly mixed by keeping the flasks on a rotary shaker at 150 rotations per minute for 30 min. The mixture was centrifuged at 9000 rotations per minute for 10 min at 4° C. The supernatant was collected and used for enzyme assay.

Enzyme assay:

Enzyme activity was determined by a dinitrosalicylic acid (DNS) method. This method works on the concentration of glucose (for pectinase, amylase and cellulase) and {N-acetyl-β-d-glucosamine (NAG) (for chitinase)} which is released as a result of enzymic action. The 2ml reaction mixture contained 0.5 ml of 0.5% colloidal chitin (for chitinase), pectin (for pectinase), starch (for amylase) and carboxy methyle cellulose (for cellulase) in phosphate buffer saline (pH 7.4), 0.5 ml crude enzyme extract and 1ml distilled water. The well vortexed mixture was incubated in a water bath shaker at 37° C for 30 min. The reaction was arrested by the addition of 3ml DNS reagent followed by heating at 100° C for 10 min with 40% Rochelle's salt solution. The colored solution was centrifuged at 10,000 rotations per minute for 5 min and the absorption of the appropriately diluted test sample was measured at 530 nm using UV spectrophotometer Analytic zenna, Germany) along with substrate and enzyme blanks. One unit (U) of the enzyme activity is defined as the amount of enzyme that is required to release 1µmol of N-acetyl-β-d-glucosamine (for chitinase) and glucose (for pectinase, cellulase and amylase) per minute.

RESULTS

Fig. 1: Enzyme production on petri plate by different isolates

Quantification of amylase production by isolate SA 1:

Table 1: Amylase production by isolate SA 1

No. of days	O.D	Enzyme activity (µg/ml/min)	Enzyme activity (µM/ml/min.)
1	2.361	11.149	0.061
2	3.417	16.068	0.088
3	3.873	18.512	0.102
4	4.984	23.785	**0.131**
5	3.485	16.67	0.092

Amylase production by isolate SA 1 was observed maximum on day 4 then it gets decreased. Maximum enzyme activity was 0.131 µM/ml/min.

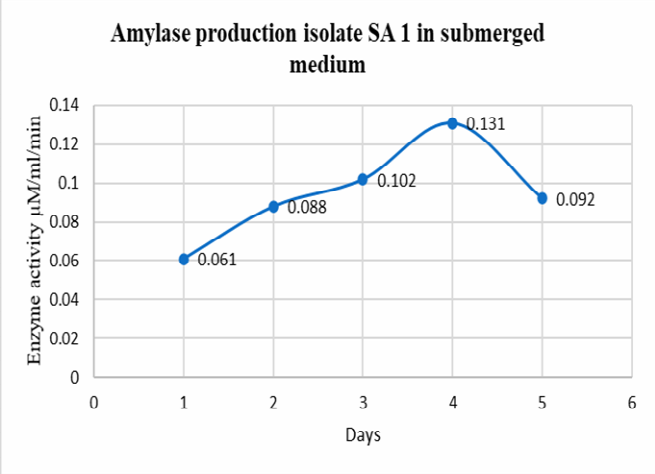

Fig. 2 : Amylase production by isolate SA 1

Quantification of cellulase production by isolate SA 1:

Table 2: Cellulase production by isolate SA 1

No. of days	O.D	Enzyme activity (µg/ml/min)	Enzyme activity (µM/ml/min.)
1	0.8369	4.102539	0.022772
2	0.9738	4.752294	0.026378
3	1.3959	6.755665	0.037498
4	1.5032	7.264932	0.040325
5	1.5903	7.678326	0.042619
6	1.6721	8.066565	0.044774
7	1.6923	8.162439	0.045307
8	1.7193	8.290586	0.046018
9	1.7421	8.398799	0.046619
10	1.755	8.460025	0.046958
11	1.879	9.048554	0.050225
12	1.829	8.811244	0.048908

For quantification of cellulase activity, isolate SA 1 was observed for 12 days, it was found that at 11 days of incubation enzyme activity was maximum with 0.050225 µM/ml/min. Secretion of enzyme start after 24 hour of incubation and it gets increased up to 11 days but then it start decreasing.

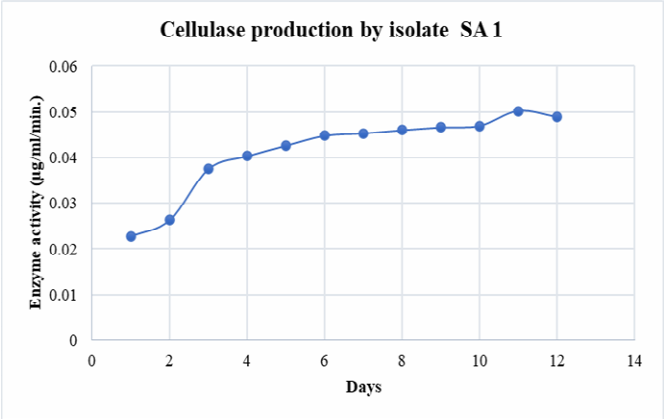

Fig 3: Cellulase production by isolate SA 1

Quantification of pectinase production by isolate SA 2:

Table 3: Pectinase production by isolate SA 2

No. of days	O.D	Enzyme activity (µg/ml/min)	Enzyme activity (µM/ml/min.)
1	2.9314	14.04346	0.07795
2	3.2994	15.79006	0.087645
3	3.4521	16.5148	0.091667
4	2.9149	13.96514	0.077515
5	0.8737	4.277199	0.023741

Isolate SA 2 showed maximum activity with 0.091667 µM/ml/min. on third day of incubation.

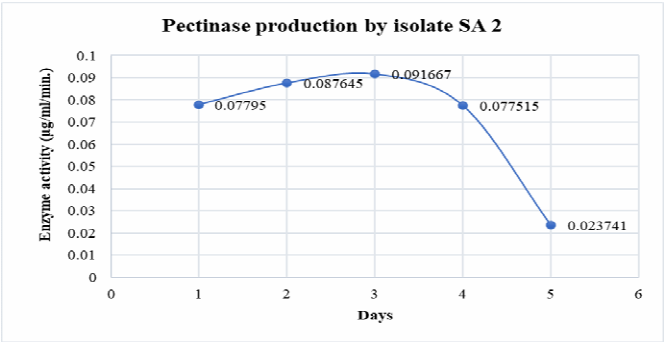

Fig 4: Pectinase production by isolate SA 2

Quantification of chitinase production by isolate SA 3:

Table 4: Chitinase production by SA 3 in submerged medium

No. of days	O.D	Enzyme activity (µg/ml/min	Enzyme activity (µM/ml/min.)
1	0.2972	1.598551	0.007226
2	0.3223	1.719807	0.007775
3	0.5729	2.930435	0.013247
4	0.8732	4.381159	0.019805
5	1.8958	9.321256	**0.042138**
6	0.9823	4.908213	0.022188
7	0.8356	4.199517	0.018984

Chitinase production was assayed in submerged medium and maximum activity was found after incubation of five days with 0.042138 µM/ml/min.

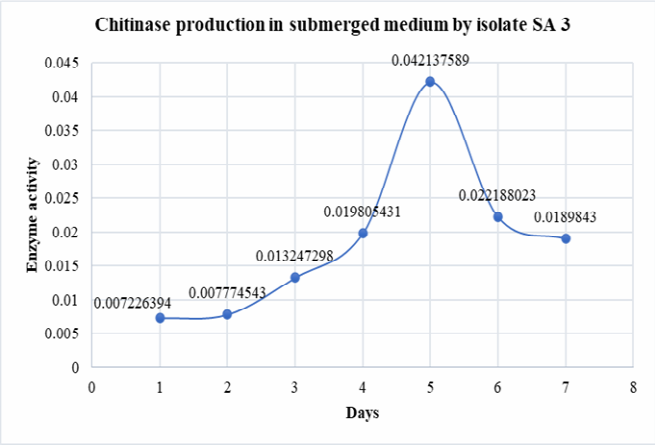

Fig 5: Chitinase production by isolate SA 3 in submerged medium

Table 5: Chitinase production in solid state fermentation by isolate SA 3

No. of days	O.D	Enzyme activity (µg/ml/min	Enzyme activity (µM/ml/min.)
1	0.0823	0.560386	0.002533
2	0.1342	0.811111	0.003667
3	0.7652	3.85942	0.017447
4	1.7682	8.704831	0.039351
5	3.8892	18.95121	0.085671
6	2.9729	14.52464	0.06566
7	1.2527	6.214493	0.028093

Chitinase production by isolate SA 3 in solid state fermentation was also observed and it was found that it have more enzyme activity in comparison of submerge medium. Maximum enzyme activity was 0.085671 µM/ml/min. on five days of incubation.

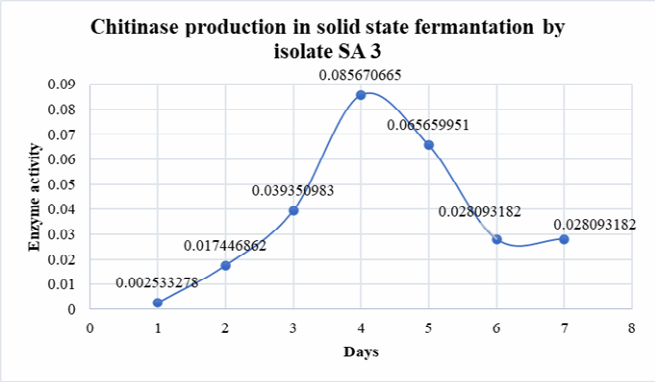

Fig. 6: Chitinase production by isolate SA 3 in submerged medium

DISCUSSION

Search of new microorganism for enzyme production are increasing around the world. Actinomycetes constitute a potential source of biotechnologically interesting substances, which make it one of the most investigated group. Due to formation of stable mycelia, streptomycetes have ability to secrete an array of different extracellular enzymes including cellulose, amylase, pectinase and chitinase. The purpose of this experiment was qualitative and quantitative study of extracellular enzyme production by endophytic actinomycetes from *Syzygium cumini.* Chitinase (EC 3.2.1.14) is an enzyme which can hydrolyze chitin to its oligomeric, dimeric and monomeric component. The enzyme found in numerous bacteria, fungi, insect, plant and animal involves in natural protection mechanism. Many bacteria and fungi containing the chitinolitic enzyme convert chitin into carbon and nitrogen that can serve as energy source. Chitinolitic bacteria are typically detected and screened through the production of clearing zones on chitin containing agar as selective medium. Chitinases have many industrial and agricultural applications such as preparation of chitooligosasccharides, biocontrol of plant pathogenic fungi, and production of single-cell protein. Varied enzyme production may result in new biochemical characteristics and in part be responsible for the inherent biodiversity of endophytic micro-organisms. Chitinase production was evaluated in this study and it was found that this isolate (SA 3) was very good producer of chitinase. Chitinase production was assayed in submerged medium with maximum activity of 0.042138 µM/ml/min and in solid state fermentation medium, maximum enzyme activity was 0.085671 µM/ml/min. This isolate can be used as biocontrol agent for controlling plant pathogenic fungi. It was also observed that this isolate have produced more enzyme in solid state fermentation in comparison to submerged medium. Solid state fermentation (SSF), a process that occurs in the absence or near absence of water, has been used for the production of various high value added products such as enzymes and other organic components. SSF allows for the production of enzymes with higher activity and stability with lower water and energy demands. Additionaly, from the environmental and economic perspectives, the main advantage of SSF is related to lower volume of effluent produced, compared to SmF, and the possibility of carrying out the process under non-sterile conditions. Among the lytic enzymes evaluated as a source of bio control agents, chitinases have been studied largely because these enzymes are produced by a variety of endophytic micro-organisms (El-Tarabily and Sivasithamparam, 2006). A correlation between chitinolytic and antagonistic activities was observed for fungi but not oomycetes, which possess cellulose as their main cell wall compound. Chitinolytic activity has been implicated in the biocontrol activity of several bacteria, including *Streptomyces* spp. However, for many biocontrol systems, a direct statistical correlation for the role of chitinase is lacking. it was reported that a maltotriose forming amylase from *Streptomyces griseus* and a maltotetraose-forming amylase from *Pseudomonas stutzeri* could transfer maltotriose and maltotetraose to p-nitrophenyl α-glucopyranoside, respectively (Usui and Murata, 1988). In this experiment, we quantify amylase production by isolate SA 1, enzyme activity was maximum with 0.131µM/ml/min. Pectin and pectinase have a wide global market. Pectinases which hold a share of 25% in the global sales of food enzymes are widely used in the beverage industry due to their ability to improve pressing and clarify cation of concentrated fruit juices. Isolate SA 2 showed maximum pectinase activity with 0.091667 µM/ml/min. on third day of incubation. Bacterial cellulolysis has recently gained importance as potential source for development of commercial processes because of its high growth rate, high genetic variability, adaptability and high amenability to genetic manipulation. *Streptomyces* sp. substantially hydrolyzing crystalline cellulose like avicel have been reported. Several actinomycetes reported in the literature have been able to produce cellulolytic enzymes, most of them belonging to the genus *Streptomyces*, such as *S. antibioticus, S. flavogriseus, S. lividans, S. Reticuli,* and *S. hygroscopicus*. Most of the *Streptomyces* cellulases are active against amorphous or soluble cellulose; therefore, being able to produce endoglucanases but unable to degrade microcrystalline cellulose, which requires the presence of exoglucanase. In our experiment we found maximum cellulase activity with 0.050225 µM/ml/min.

CONCLUSION

In this study, three entophytic actinomycetes from *Syzygium cumini* were selected for quantification of four extracellular enzymes (chitinase, cellulase, amylase and pectinase) production. Selected isolates were SA 1, SA 2 and SA 3; SA 1 was selected for quantification of cellulase and amylase production in submerged medium. SA 2 was selected for quantification of pectinase production in submerged medium while SA 3 was selected for quantification of chitinase production in submerged medium and solid state fermentation medium. Amylase production by isolate SA 1 was observed maximum on day 4 with maximum enzyme activity of 0.131 µM/ml/min. Isolate SA 2 showed maximum pectinase activity with 0.091667 µM/ml/min. on third day of incubation. Cellulase activity by isolate SA 1 was observed maximum after 12 days with 0.050225 µM/ml/min. Chitinase production in submerged medium by isolate SA 3 was maximum activity on fifth days with 0.042138 µM/ml/min while in SSF medium, maximum enzyme activity was 0.085671 µM/ml/min.

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