International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 25-07-2021, Accepted: 08-08-2021, Published: 25-08-2021
Volume 6, Issue 4, 2021, Page No. 907-913

Amylolytic potentiality of fungi isolated from agricultural wastes

Jeyabharathi S*, Jeenathunisa N, Aruna V
PG and Research Department of Microbiology, Cauvery College for Women (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Abstract
The biocatalysts enzymes are used in multifarious ways in different field. The applications into four broad categories: *(i)* Therapeutic uses, *(ii)* Analytical uses, *(iii)* Manipulative uses, and *(iv)* Industrial uses. The production of enzymes would be a challenging task hence mass production of enzymes from agricultural wastes would be a emerging trend now. In this present study Agricultural waste Banana stem were used as a substrate from which amylolytic fungal isolate *Aspergillus ustus* Asp-2910 was subjected to submerged and solid-state fermentation of Amylolytic α-amylase enzyme. Amount of α-amylase liberated by identified mould was quantified by estimating the amount of reducing sugars produced when specified quantity of starch was hydrolyzed after incubation at 40°C with known concentration of enzyme solution. The specific activity was recorded as 4.731 IU/mg for amylase enzyme produced by *Aspergillus ustus* Asp-2910 in Solid State fermentation and submerged fermentation the activity recorded as 52 IU/mg this results showed the better production of amylolytic enzyme facilitated by Banana stem through submerged cultivation method than solid state method.

Keywords: fermentation, agricultural wastes, amylolytic α-amylase, solid state fermentation, submerged fermentation

Introduction

Enzymes are Protein that catalyse the biochemical reaction in living cell. They are high molecular weight compounds made up of amino acids link together by peptide bonds [1].Amylase are enzyme that catalyse the hydrolysis of starch into sugars.Amylase present in the slaiva of humans and some other mammals.It is mainly useful applications in the food, brewing, textile, detergent, pharmaceutical industries [2]. α-amylase is an oligosaccharide endoglycosidase, an enzyme that cleaves an internal glycosidic bond within a poly or oligosaccharide [3]. α –amylase can be found in a variety of body fluid. If any injury in Pancreas the rate of α-amylase could beincreased. So that the increased amount of α-amylase used to identifyingthe Pancreatitis. And it also used to break the internal blood clots, To dissolve the hardening of walls of blood vessels, To dissolve the wound swelling to promote healing [4]. Amylase is an unique enzyme that plays a incomparable role in the field of microbial biotechnology. It is produced mainly from bacteria and fungalsources and is industrial sectors with top-down and bottom-up approaches are currently focusing on improving microbial amylase production levels by implementing bioengineering technologies and environment-friendly technologies [5]. Here in, the importance of microbial amylase is discussed along with its production methods from the laboratory to industrial scales [6]. The further support of energy consumption studies, such as those on thermodynamics andpinch technologyhas hastened the large-scale production of the enzyme. These enzymes randomly cleave internal glycosidic linkages in starch molecules to hydrolase them yield dextrins and oligosaccharides [7].Among amylases α-amylase is in maximum demand due to the wide range of applications in the industrial level [8].With consumers growing increasingly aware of environmental issues, industries find enzymes as a good alternative over other chemical catalysts.α –amylase can be produced by plant or microbial sources.Due to the advantages that microbial productions offers, α –amylase from microorganisms has been focused upon and preferred to other sources for production [9, 10].The ubiquitous nature, ease of production and broad spectrum of applications make α-amylase an industrially important enzyme.This report focuses on the microbial production of α –amylase and its applications [11].Alpha amylase is an oligosaccharide endoglycosidase, an enzyme that cleaves an internal glycosidic bond within a poly or oligosaccharide [12]. In the case of alpha amylase, it is the 1,4 linkage between two glucose moieties, cleaving the C-O bond between the C1 carbon and the oxygen, although which 1,4 linkage is cleaved is random:

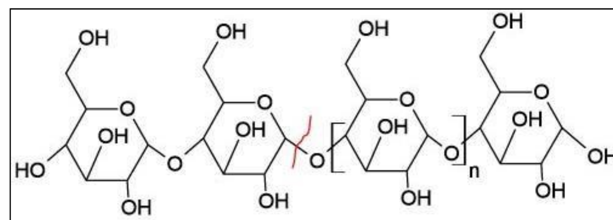


Fig 1

In the present study, characterization of amylase producing *Aspergillus ustus asp 2910* isolation and optimization of medium, cultural condition of amylase enzymes production, extraction and partial purification of extra cellular enzymes. The α–amylase of *Aspergillus ustus asp 2910* had the pH optima ranged 4-6 and temperature optima ranged at 30^0-40ºC [13]. The purpose of this work is to study the optimized cultural conditions for the production of amylase by *Aspergillus ustus asp 2910*in solid state and submerged liquid fermentation.

Materials and Methods

Sample collection

Soil sample were collected from near Cauvery College for women then stored in air tight container for further processing.

Substrate preparation

Banana stem collected from agricultural field, Tiruchirappalli District. Collected stems cut in to small pieces around 1 cm length and then it has been dried carefully without further fungal contamination. Dried banana stem pieces ground finely sorted then the powder stored in air tight container for further processing.

Isolation of fungi

Soil sample were serially diluted. The medium was prepared using potato dextrose agar And this was pured into the petriplates. Diluted soil samples were dropedinto the PDA agar plates Using the L-rod the soil sample was spreaded. And the plates were incubated aerobically at 29^0C for 4 days [14].

Identification of selected fungal isolate

Identification of fungal isolates was performed based on their Morphocalogical, Physiological, biochemical and 18S rRNA sequencing methods.to confirm the identities of the isolates, PCR amplification and sequencing of the 18 S rRNAgene were performed.

Phylogenetic analysis

The acquired sequences were used for a gene homology search, with the18S rRNA sequences available in the public databases from BLAST. Using the CLUSTAL-X multiple sequence alignment program, the18S rRNA sequence of the isolated strains were aligned with sequence related organisms obtained from Gen Bank [15]. Phylogenetic analysis was performed with DNA MEGA 4, and a sequence distance was calculated and the phylogenetic trees was constructed. The partial 18S rRNA sequence of the isolated strains were deposited in Gen Bank.

Determination of amylolytic potentiality of fungi isolates

In 1000 ml distilled water was taken in the sterile conical flask.A modified medium consisting of soluble starch 20 gm, peptone 2 gm, ammonium sulphate 3 gm, potassium dihydrogen phosphate 1gm, sulphate heptahydrate 0.3gm and calcium chloride 0.3 gm were sterilized. The spore suspension containing 10^5 spores/ml of 7 day old culture of each isolate was aseptically introduced into the fermented medium. This was incubated at the room temperature of 60-65^0C. And at the intervals of 5 min sample was drawn and tested with 2% of iodine solution. Failed to form of blue colouration was used the point at which each organism exhibited amylolytic property. In addition, each pure isolate was transferred with sterile inoculation loop on sterile starch agar plate and incubated for 5-7 days at room temperature of 30^0C. Each colony was checked for colony halo-forming zone whose diameter was measured [16].

Submerged fermentation method for amylase production

The cultivation medium containing dry waste 2gm, peptone 0.2gm, ammonium sulphate 0.3gm and potassium dihydrogen phosphate 0.1gm, magnesium sulphate heptahydrate 0.03gm and calcium chloride 0.03gm was added in the 100 ml of distilled water. The medium was sterilized and allowed to cool. And inoculated with 7 day old culture fungal suspension containing 10^5 spores/ml. This was followed by incubation at room temperature at 30^0C for 6 days [17].

Preparation of crude enzyme

Harvested cultures were cooled at 4 degree C for 30 min, centrifuged at 12000, x g, and supernatant separated and used as crude enzyme.

Solid state fermentation method for amylase production

Medium containing 1 gm dry agricultural waste and 50 ml distilled water used. Dissolved in distilled water peptone 0.1gm, ammonium sulphate 0.15 gm, Potassium dihydrogen phosphate 0.05 gm, magnesium sulphate heptahydrate 0.015 gm and calcium chloride 0.015 gm. The wet medium was then sterilized, cooled and inoculated with a spore suspension of approximately 10^5 spores/ml prepared from 7 days old fungal cultures [18].

Preparation of crude enzyme

Following incubation, each waste medium was mixed with distilled water in the ratio of1:9 (w/v) and agitated at 30 degree C for 30 min.The mixture was subsequently cooled, centrifuged and supernatant for enzyme activities.

Assay for amylase

The method fused involved estimating the presence of reducing sugar quantitatively by the activity of each enzyme on buffered starch. The substrate for assay was 0.5 ml of 0.05 ml soluble starch, buffered with 0.02 ml of 0.1M sodium acetate (pH 5.6) Crude enzyme extract (0.3ml) was added to the mixture, mixed and incubated at 40^0 C for 30 min in a water bath. DNSA method as used.One ml DNSA solution was added to the mixture and boiled for5 min. Four ml of distilled waterwas introduced cooling before absorbance is read at 540 nm in UV/Visspectrophotometer [19].

Spectrophotometry

A spectrophotometer is used to find the amount of radiant energy absorbed or transmitted by molecules in a solution as a function of wavelength. The wavelength which a certain molecule can absorb energy is different and therefore it can be used to determine the concentration of a specific type of solution. By comparing the amount of light that is absorbed by the sample with known concentration a calibration curve can be plotted and by using it concentration of the unknown sample can be determined [20].

HPLC analysis

Samples were filtered through a 0.45µm Millex-HV PVDF membrane (Millipore, New Bedford, MA, USA). HPLC analyses were performed on a Shimadzu chromatographer equipped with a ternary pump (Shimadzu LC-20AT) and DAD detector (Diode Assay Detector) (Shimadzu SPD-M20A, Kyoto, Japan) and carried out on an analytical column (Phenomenex® ODS 100A 250mm × 4.60mm, 5µm) preceded by an C18 guard column also from Phenomenex. LC solutions software (version 1.25) was used for data processing. Chromatographic conditions were gradient using acetonitrile and water as mobile phase,

initially consisting of acetonitrile/water and increasing up to acetonitrile/water (in 30 minutes at a flow rate of 1mL·min^{-1}. The mobile phase was prepared daily and degassed by sonication before use. The column temperature was 25°C and the injection volume was 20μL. The UV spectra were monitored over a range of 450 to 200nm, while the chromatograms were recorded at 273 nm to detect the furan ester derivative 280nm [21].

Results and Discussion

Macroscopic observation

The fungi isolation was done by the use of potato dextrose agar. The colonies were found on the plates. Fungi are filamentous, composition and ubiquitous fungus found in nature. Its conditions are downy to powdery in texture.The surface colour may vary depending on the species.The reverse is uncoloured to pale yellow in most of the species (Fig: 2).

Fig 2: Colony morphology of fungi

Isolation of fungi

Every single colony of different shapes and colours appeared on the PDA plate after the first incubation was subcultured and maintained on another new PDA plate. Nine single colonies were successfully isolated on a new plate and were presented. The isolated plates were subjected to identification, The isolates were identified by Lactophenol cotton blue staining by morphological identification for genus level. *Aspergillus*is a dominant species found in the soil sample then the isolates were subjected to 18s rRNA sequencing for species level identification results expected that could be a *Aspergillus ustus*Asp-2910

Micrscopic observation

In the microscopic observation hyphae are septate and hyaline.The conidiophores originate from the basal foot cell and terminate in a vesicle at the apex.Covering the surface of vesicle are flask shaped phialides which are either uniserate or bioserate.Over the phialides are the round conidia (2-5 μm in diameter) forming radial chains. It grows well on potato Dextrose agar and sabouraud dextrose agar (Fig: 3).

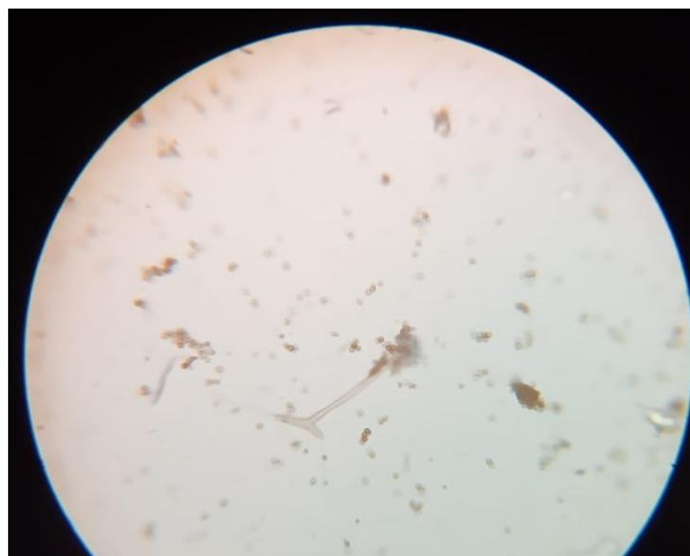


Fig 3: Microscopic appearance of fungi

Molecular characterization of fungal isolate

The acquired sequences were used for a gene homology search, with the18 S rRNA sequences available in the public databases from BLASTN 2.9.0+ Using the multiple sequence alignment program, the18 S rRNA sequence of the isolated strains were aligned with sequence related organisms obtained from GenBank [22] (Thompson *et al*., 1997).Phylogenetic analysis was performed with DNA MEGA 4, and a sequence distance was calculated and the phylogenetic trees was constructed. From the above results the identified Species as Aspergillus ustus isolate Asp-2910 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence Sequence ID: KY203997.1 Length: 680 Number of Matches: 3.The partial 18 S rRNA sequence of the isolated strains were deposited in GenBank (Fig: 4)

Fig4: Blastn 2.9.0+ database

Amylolytic potentiality of fungi

The fungal speciesgrown in the medium containing peptone, ammonium sulphate and magnesium sulpahte heptahydrate, potassium dihydrogen phosphate, calcium chloride. And the medium was drawn in a flask and it was flooded with 0.2% gram's iodine. There was no colour change in the medium (fig: 5). It indicates the starch is hydrolysed by the amylase.Enzyme occurring in every living cell, hence in all microorganism.Each single strain of organism produces a large number of enzyme hydrolyzing, oxidizing or reducing and metabolic in nature. An extremely important use for fungal amylase is inconversion of partially acid hydrolyzed starch to sweet syrups.Acid hydrolysis is a random action whereas enzymic hydrokysis is a patterned one.To meet the demand of industries, Low cost medium is required for the production of α-amylase.Both solid state fermentation (SSF) and Submerged fermentation (SmF) could be used for the production of α-amylase, although traditionally these have been obtained from submerged culture because of ease handling and greater control of environmental factors such as tempertature and pH, Conclusively, amylase can be produced for industrial purpose from *Aspergillus ustus*Asp-2910grown in banana stem. More recently, many authors have presented good results in developing α-amylase purification technique, which enable applications in pharmaceutical and clinical sectors which require highpurity amylases.

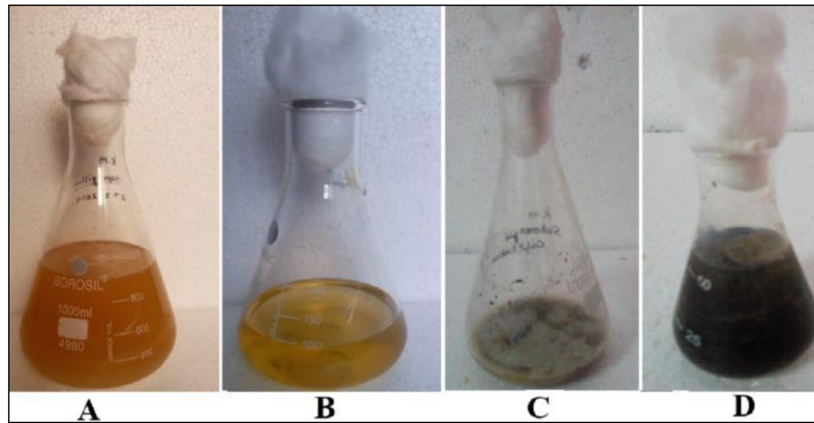


Fig 5: A. Amylolytic potentiality of fungi, B. starch hydrolyzation, C. submerged fermentation, D. solid-statefermentation

Starch hydrolysation

Activity on starch hydrolysis by *Aspergillus ustus*Asp-2910was measured at different days interval growing culture at 30 °C.*Aspergillus ustus*Asp-2910was found to hydrolyse higher amount of starch in culture. Starch hydrolysis wasnoticed that the medium was flooded with 0.2% gram's iodine solution. There was no colour change in the medium. It wasrecorded in six and seven days old culture forthe fungal species. Higher activitywas recorded for enzyme secreted by *Aspergillus ustus*Asp-2910.Maximum activity was recorded with enzyme extract of 6th day old culture for both fungal species. A reduction in activity was noticed with enzyme extract of 7th days onwards old culture.

Maximum production of α-amylase was shown by *Aspergillus ustus*Asp-2910in 9 day old culture. It was noticed that from 5th to 9th days of incubation there was steady increase in production of α-amylase which begin to decline only after 9 day old culture.

Submerged fermentation

In this fermentation technique *Aspergillus ustus*Asp-2910was found to be the best amylase producer and specific activity of amylase produced by this strain was 52 U/mg in Submerged fermentation. Similar values of enzyme production [23] for Aspergillus *ustus* isolates JGI24 and GCB –34 have been reported. So, this potential strain was selected for further optimization of culture conditions.

Solid state fermentation

Banana stem were used as solid substrates for SSF. After inoculation and incubation for six days at room temperature with pH 7, the enzyme was extracted using phosphate buffer and was estimated for the protein content and the enzyme activity. The specific activity was recorded as 86 U/mg for amylase enzyme produced by *Aspergillus ustus* Asp-2910. High amylolytic activity of *Aspergillus ustus* Asp-2910 in biomass production found that *Aspergillus ustus* Asp-2910 was superior to other species of *Aspergillus* and strains of fungi in biomass yield from agricultural waste [24]

UV/Visspectroscopy

Spectrophotometer analysis is extremely selective, sensitive and colored substances absorb in the visible range. Variation of the colour of the solution with charge in concentrations from the basis of spectrophotometer analysis. From the UV/ Vis spectral analysis of hydrolysis of starch is detect by the UV spectroscopy (fig: 6). The crude extract of solid state and submerged fermentation culture were undergo the analysation. Sample 1 concentration was submerged fermentation and sample 2 was considered as solid state fermentation crude extract. Sample 1 range at 0.674 and sample 2 was 0.019. And the concentration of the samples on the % of assay at 2543.6 for submerged fermentation and 8000 for solid state fermentation.

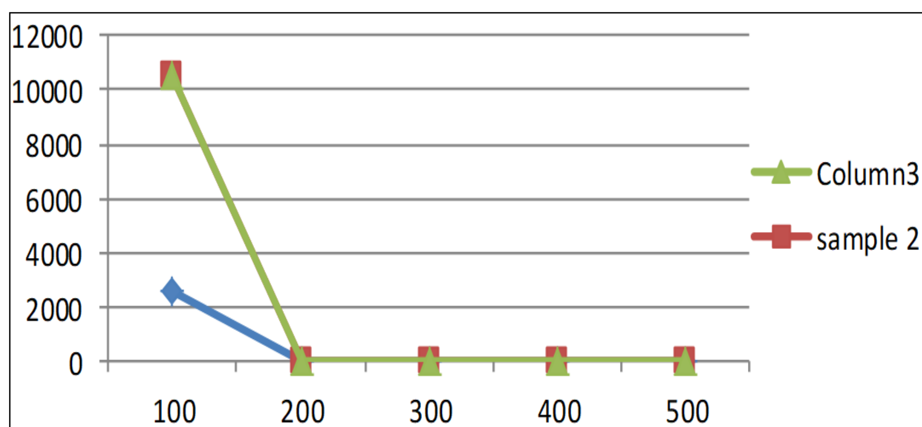
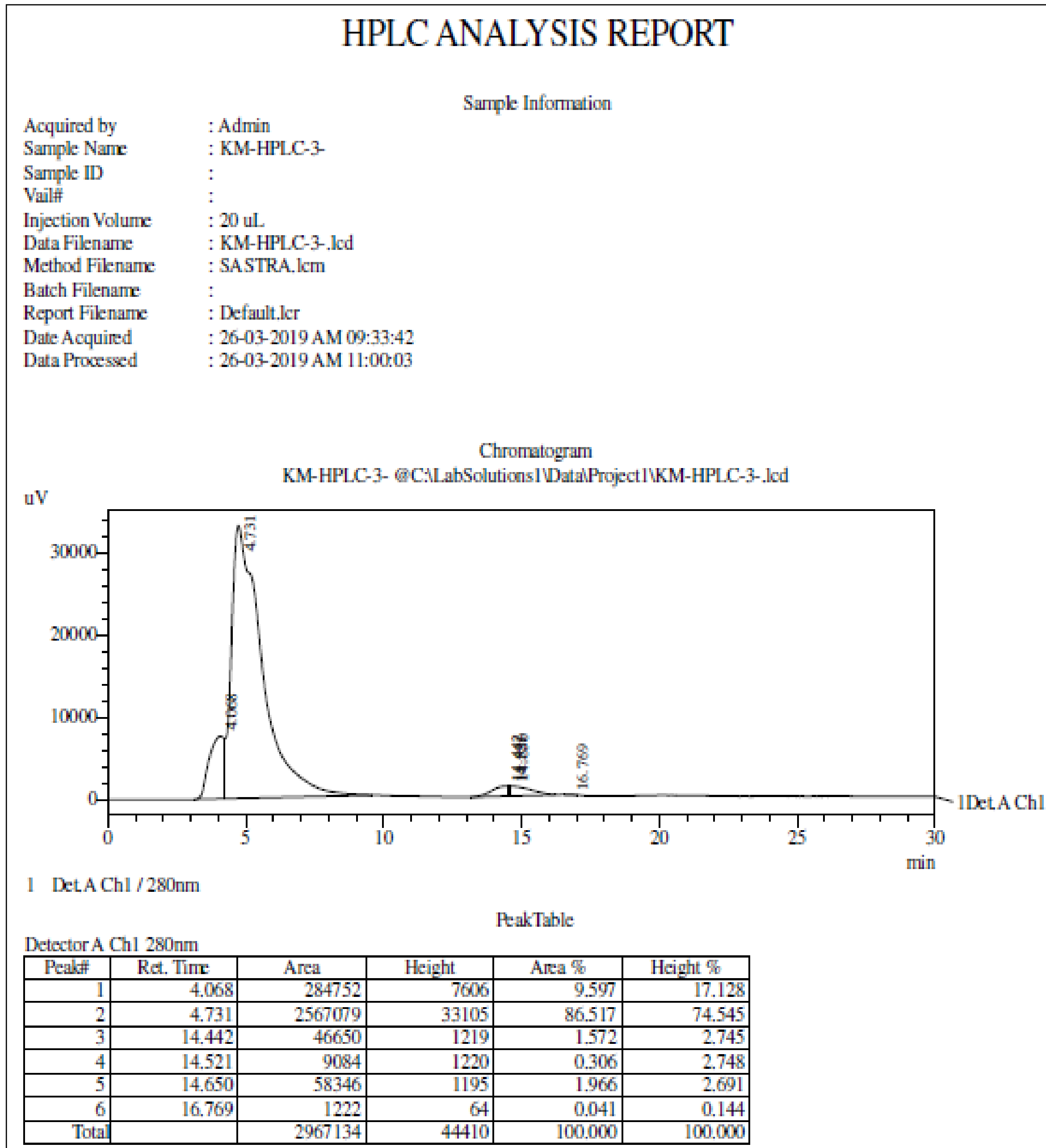


Fig 6: Graphical analysation of (solid-state and submerged) crude extrate in uv spectrophotometer

HPLC analysation

HPLC analysation was done on the crude extract of solid state and submerged fermentation crude extract. Graphical and table analysation was shown in (fig: 6). The value of crude enzyme from this results dominate amylolytic compound at the range of 4.731from solid state fermentation.



HPLC ANALYSIS REPORT

Sample Information

Acquired by : Admin
Sample Name : KM-HPLC-3-
Sample ID :
Vail# :
Injection Volume : 20 uL
Data Filename : KM-HPLC-3-.lcd
Method Filename : SASTRA.lcm
Batch Filename :
Report Filename : Default.lcr
Date Acquired : 26-03-2019 AM 09:33:42
Data Processed : 26-03-2019 AM 11:00:03

PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.068	284752	7606	9.597	17.128
2	4.731	2567079	33105	86.517	74.545
3	14.442	46650	1219	1.572	2.745
4	14.521	9084	1220	0.306	2.748
5	14.650	58346	1195	1.966	2.691
6	16.769	1222	64	0.041	0.144
Total		2967134	44410	100.000	100.000

FIG 6: HPLC analysis chromatogram

Conclusion

This study has revealed that the capable of producing α-amylase on banana stem under solid state fermentation. The fungi *Aspergiullus* was isolated from soil. From the genus level processing *Aspergillus ustus* Asp-2910 were identified. There is no colour change in the fermentation medium after adding Grams Iodine conclude the identified fungi was Amylase producer [24]. And then the efficiency of the fungal amylase production was studied by solid state fermentation process of α-amylase is about 4.731IU/ml using the banana stem wastes. Under this study the identified Fungi *Aspergillus ustus* Asp-2910 showed that the good producer of α-amylase under solid state fermentation [25]. Hence this method might be useful for further mass cultivations and also to Satisfied the need of α-amylase [26]. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. It supports normal immune response, resist to swelling and redness, Inhibits Cell growth, Normalize blood sugar.An implication and clinical application of amylase Hyperamylasemia (Pancreatic, salivary, macroamylasemia and combinations) infection is proposed [27].

References

1. Asoodeh A, Chamani J, Lagzian M. A novel thermostable, acidophilic alpha-amylase from a new thermophilic "*Bacillus* sp. Ferdowsicous" isolated from

Ferdows hot mineral spring in Iran: Purification and biochemical characterization. Int J Biol Macromol,2010:46:289-297.
2. Adeniran AH, Abiose SH. Amylolytic potentiality of fungi isolated from some Nigerian agricultural wastes. African Journal of Biotechnology,2010:8(4):667-672.
3. Adney B, Baker J. Measurement of cellulase activities. NREL Analytical Procedure, National Renewable Energy Laboratory, Technical, Report NREL/TP, 2010, 510-42628.
4. Akpan I, Bankjole MO, Adesermowo AM, Lantunde-Data. Production of α-amylase by *Aspergillus niger* in a cheap solid medium using rice bran and agricultural material. Trop.sci,1999:39:77-79.
5. Alva S, Anupama J, Salva J, Chiu YY, Vyshali P, Shruthi M *et al*. Production and characterization of fungal amylase enzyme isolated from *Aspergillus sp.* JGI 12 in solid state culture. African journal of Biotechnology,2007:6:576-581.
6. Aneja KR. Experiments in Microbiology, plant pathology and biotechnology, Fourth edition, New Age International (p). Ltd., publishers, New Delhi, 2007, 607-612.
7. Anupama, Ravindra P. Studies on production of single cell protein by *Aspergillus niger* in solid state fermentation of rice bran. Braz. Arc. Biol. Tec,2011:44:79-88.
8. Vu VH, Pham TA, Kim K. Improvement of fungal cellulase production by Mutation and optimization of solid state fermentation. *Mycobiology*,2011:39(1):20-25.
9. Mrudula S, Kokila R. Production of Thermostable a-amylase by *Bacillus cereus* MK in solid state fermentation: partial purification and characterization of the enzyme. Internet Journal of Microbiology,2010:8(1)48:17-25.
10. Zia MA, Khalil-Ur-Rahman K-U, Sheikh MA, Khan IA. Chemically treated strain improvement of *Aspergillus niger* for enhanced production of glucose oxidase. International Journal of Agriculture and Biology,2010:12(6):964-966.
11. Singh RK, Mishra SK, Kumar N. Optimization of *α*-amylase production on agriculture byproduct by *Bacillus cereus* MTCC 1305 using solid state fermentation. Research Journal of Pharmaceutical, Biological and Chemical Sciences,2010:1(4):867-876.
12. Vardhini RDS, Reddi Naik B, Neelima M, Ramesh B. Screening and production of *α*-amylase from *Aspergillus niger* using zero, value material for solid state fermentation. International Journal of Pharmacy and Pharmaceutical Sciences,2013:5(1):55-60.
13. Bano S, Qader SAU, Aman A, Syed MN, Azhar A. Purification and characterization of novel *α*-amylase from *Bacillus subtilis* KIBGE HAS. AAPS Pharm Sci Tech,2011:12(1):255-261.
14. Hashemi M, Razavi SH, Shojaosadati SA, Mousavi SM, Khajeh K, Safari M. Development of a solid-state fermentation process for production of an alpha amylase with potentially interesting properties. J Biosci Bioeng,2010:110:333-337.
15. Ur Rehman H, Qader SAU, Aman A. Polygalacturonase: production of pectin depolymerising enzyme from bacillus licheniformis KIBGE IB-21. Carbohydr Polym,2012:90:387-391.
16. Bück A, Casciatori FP, Thoméo JC, Tsotsas E. Model-based control of enzyme yield in solid-state fermentation. Procedia Eng,2015:102:362-371
17. Kirankumar V, Ravi Sankar N, Shailaja R, Saritha K, Siddhartha E, Ramya S *et al*. Purification and Characterization of α-Amylase Produced by Aspergillus niger Using Banana Peels, Journal of Cell and Tissue Research,2011:11(2)2775-2780.
18. Mohamed SA, Al-MalkiL AL, Khan JA, Kabli SA, Al-Garni SM. Solid state production of polygalacturonase and xylanase by *Trichoderma* species using cantaloupe and watermelon rinds. J Microbiol,2013:51:605-611.
19. Kumar A, Duhan JS. Production and Characterization of Amylase Enzyme Isolated from *Aspergillus niger* MTCC-104 Employing Solid State Fermentation, Int. J. Pharma and Bio Sciences,2011:2(3):250-258.
20. Panwar D, Srivastava PK, Kapoor M. Production, extraction and characterization of alkaline xylanase from Bacillus sp. PKD-9 with potential for poultry feed. Biocatal Agric Biotechnol,2014:3:118-125.
21. Rajashri DK, Anandrao RJ. Optimization and scale up of cellulase-free xylanase production in solid state fermentation on wheat bran by *Cellulosimicrobium* sp. MTCC 10645. Jordan J Biol Sci,2012:5:289-294.
22. Mrudula S, Reddy G, Seenayya G. Effect of substrate and culture conditions on the production of amylase and pullulanase by thermophilic *Clostridium thermosulforegenes* SVM17 in solid state fermentation. Malays J Microbiol,2011:7:19-25.
23. Unakal C, Kallur RI, Kaliwal BB. Production of α-amylase using banana waste by *Bacillus subtilis* under solid state fermentation. Eur J Exper Biol,2012:2:1044-1052.
24. Arora S, Rani R, Ghosh S. Bioreactors in solid state fermentation technology: design, applications and engineering aspects. J Biotechnol,2018:269:16-34
25. Ashok A, Doriya K, Rao DRM, Kumar DS. Design of solid state bioreactor for industrial applications: an overview to conventional bioreactors. Biocatal Agric Biotechnol,2017:9:11-18
26. Behnam S, Karimi K, Khanahmadi M, Agriculture I, Resources N, Salimian Z. Optimization of xylanase production by Mucor indicus, Mucor hiemalis, and Rhizopus oryzae through solid state fermentation. Biol J Microorg,2016:4(16):1-10
27. Biz A, Finkler ATJ, Pitol LO, Medina BS, Krieger N, Mitchell DA. Production of pectinases by solid-state fermentation of a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor. Biochem Eng J,2016:111:54-62
28. Abeywickrama CJ, Wansapala J. Review of organic and conventional agricultural products: Heavy metal availability, accumulation and safety. International Journal of Food Science and Nutrition. 2019;4(1):77-88.