Optimization of Amylase Production by *Aspergillus flavus* Using Agro Waste as Substrate

BY

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Reg.No. 19PBO005

A Dissertation submitted to the

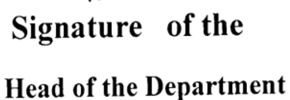
Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore – 641043.

In Partial Fulfillment of the Requirements for the Degree of

DEGREE OF MASTER OF SCIENCE IN BOTANY

MAY 2021



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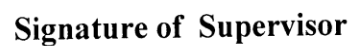

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Sprout peel waste as Substrate

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ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

First and foremost I wish to thank **God Almighty** for endowing the investigation with immense blessings which helped to overcome the hurdles, paving way for the successful completion of the study.

I express my thanks **to Dr. T.S. Avinashilingam Ayya Avl**, the Founder and First Chancellor, **Dr. Rajammal P. Devadas Amma Avl**, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity to undertake the present research programme.

I gratefully acknowledge honourable **Dr. T.S.K. MeenakshiSundaram Anna Avl**, **late Padma Shri. Dr. P. R. Krishnakumar**, **former Chancellor** for providing all necessary amenities for the completion of my work.

I owe my respectful gratitude and sincere thanks to **Prof. S. P. Thyagarajan, M.A., M.Phil., Ph.D.,** Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for all the amenities provided for the conduct of the project.

I place on record my heartful thanks to **Dr. (Mrs.) Premavathy Vijayan, M.Sc., M.Ed., Dip. Spl. Edn., M.Phil., Ph.D. (Avinashilingam), Vice Chancellor**, Avinashilingam Institute for Home Science and Higher Education for

Women, Coimbatore, for extending all possible help towards the completion of the study.

I wish to extend my thanks to **Dr. (Mrs.) S. Kowsalya, M.Sc., M.Phil., Ph.D. Registrar** for providing the academic needs during the study period.

My sincere thanks to **Dr. (Mrs) A. Vijayalakshmi, M.Sc., M.Phill., Ph.D., Dean**, School of Biosciences, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for the academic support given for the successful completion of the work.

My sincere thanks to **Dr. (Mrs.) M.K. Nisha, M.Sc., M.Phil., Ph.D., Assistant Professor and Head (i/c), Department of Botany**, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for the academic support given for the successful completion of the work.

I wish to place on record my deep sense of gratitude, profound thank and indebtedness to my respected guide **Dr. M.K. Nisha, M.Sc., M.Phil. Ph.D., Assistant Professor**, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her valuable guidance, learned counsel, cordial treatment, keen interest, kind encouragement and faster care rendered throughout the course of my investigation.

My sincere thanks for various help extended by all the **Staff members of Botany Department** and everybody who helped me and encouraged directly and indirectly throughout my study.

I expressed my heartfelt thanks to **my friends and Ph.D. scholars** of my Department for their help rendered throughout the course of my investigation.

On a personal note I express my deep sense of gratitude to **my parents and my sisters** for their prayer, encouragement, moral and physical assistance rendered by them throughout the course of my study.

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INTRODUCTION

CHAPTER I

INTRODUCTION

Agriculture is the back bone of our country and plays a very important role in the country's economy and also the food & nutritional security of our people. In agriculture the coconut palm, Date palm, and Palmyra palms plays an important role in growth of Indian economy and in earning foreign money. The leading coconut producing states in India are Tamilnadu, Karnataka, Andhra and Kerala, thus occupying the third largest coconut producing country in the world. But the improper management of agricultural waste generated in the process has been contributing towards mounting air, soil and water pollution. India generates about 350 million tonnes of agricultural waste every year. As per the estimates given by the ministry of new and renewable energy, this waste can generate more than 18,000 MW of power every year apart from generating green fertilizer for use in agriculture. Globally about 1.3 billion tonnes of food products for human consumption gets wasted or lost every year and about one-third of biodegradable municipal solid waste mainly comprise of domestic kitchen waste generated from households. Accumulation of agricultural residues such as stalks, stems, straws, hulls and cobs in large quantities every year results, not only, in the loss of potentially valuable material, but also can be processed to yield a number of valuable added products such as food, fuel, feed and a variety of chemicals. (Nigam *et al*., 2009). Several naturally occurring agricultural by-products such as wheat bran, coconut oil cake, groundnut oil cake, rice bran, maize bran, banana waste, etc. could be used in one or the other industrial bioprocess for the production of value added products. The agro industrial wastes, due to its high carbon content in

Agro industrial wastes, are employed as substrates in producing hydrolytic enzymes that hydrolyse glycosidic bonds and convert polysaccharides into simple sugars. Among many such enzymes, α-amylase is one of the most commercially exploited enzymes. Realizing the importance of management of waste generated during agricultural practices, initiation of further researches focusing on the management of agricultural waste together with converting it into some useful products has become the need of the hour.

Agro-industrial wastes consist of variable composition that supports the growth of microorganisms as a result of fermentation produced different valuable enzymes. These wastes are used as a raw material. The growth rate of fungi are enhanced by use of these substrates which resulted into the conversion of lignocellulosic substrate into less complicated ones by degrading action of several enzymes. One of the important enzymes, i.e., amylase, was used in starch processing industries for degradation of polysaccharides into sugar components (Nigam and Singh, 1995).

Amylases are starch degrading enzymes. They are widely in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristics of individual amylolytic enzymes. Initially the term amylases was used originally to designate enzymes capable of hydrolyzing α-1, 4 glucosidic bonds of amylose, amylopectin, glycogen and their degradation products. They act by hydrolyzing bonds, between adjacent glucose units, yielding products characteristics of the particular enzymes involved. Amylase enzymes catalyse the hydrolysis of α-1, 4-glucosidic linkages of polysaccharides to yield dextrin, oligosaccharides, maltose and D-glucose. These enzymes are classified according to the manner in which the glycosidic bond is attacked amylases hydrolyze α-1, 4 glycosidic linkage only from then non-reducing outer polysaccharide chain ends. Two major classes of amylases have been identified in

microorganisms, namely α-amylase and β-amylase. β-Amylase are extracellular enzymes that randomly cleave β-1, 4glucosidic linkages between adjacent units in the linear amylase chain and is the beast known endoamylase. β- Amylase can be divided from several sources, including plants, animals and microorganisms (Taskin and Erdal, 2010).

Amylase can be derived from several fungi, yeast, bacteria, and actinomycetes under both SmF and SSF utilizing various food and agronomic waste. At present, *Bacillus, Aspergillus, Rhizopus* and *rhizobial* isolates specified are considered to be the important sources of industrial amylase. Fungal species such as *Aspergillus, Rhizopus,* and *Penicillium* are considered to be the chief producers of amylase (Gupta et al., 2003).The microbial amylases meet industrial demands, a large number of them are available commercially and they have also almost completely replaced chemical hydrolysis of starch in starch processing industry. The major advantage of using microorganisms for the production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics.

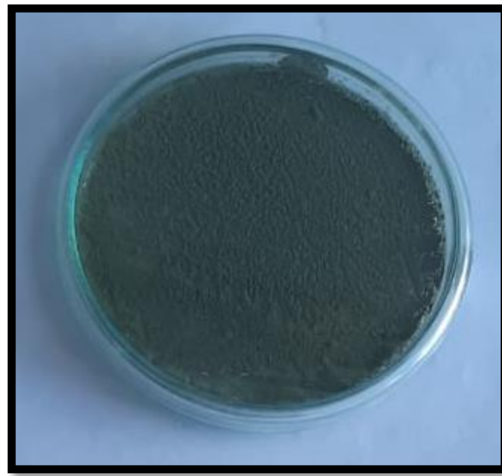
Fungal Inoculant (*Aspergillus flavus*)

Aspergillus flavus is a saprotrophic and pathogenic fungus with a cosmopolitan distribution. It belongs to the division Ascomycota of class Eurotiomycetes. *Aspergillus flavus* is a thermo-tolerant grower and thrives in hot and humid climates. It has a minimum growth temperature of 120C (540 F) and a maximum of 480C (1180 F). *A. flavus* colonies are commonly occur as powder masses of yellow-green spores on the upper surface and reddish-golden on the lower surface. Hyphal growth usually occurs by thread like branching and produces mycelium. Hyphae are septate and hyaline. The mycelium secretes degradative enzymes or proteins which can break down complex nutrients (food). Often the conidia spores the asexual spores are

produced during reproduction. The conidiophores of *A. flavus* are rough and colorless. Phialides are both uniseriate (arranged in one row) and biseriate.

PLATE – I

Plate culture of *Aspergillus flavus*



Palmyra is a long tree with a large trunk and canopy of leaves resembling a coconut tree. Palmyra palm has great economic potential and all the parts of the palm is useful in one way or the other. Palmyra palm is a 'miracle' plant due to the once wide utilization of most of its parts, such as the trunk, foliage, husk nut, sprout peel and flesh. The palm seeds germinate to produce the Palmyra sprouts.   Palmyra sprout is a tuber like vegetable more common in southern parts of India. It is high in fibre and low in Glyxemic index. They are dark or yellowish and hard to touch

and over it possess outer coverings, the peels. These peels are the leftover product after the use of the tuber. This is the abundant agrowaste used as a feed stock and deserted resulting in environmental pollution. Thus application of agricultural byproducts in enzyme production has attracted considerable attention due to low production costs and environmental friendliness

PLATE-II

Agro industrial waste (Palmyra sprout peel)

Although several investigations were employed on the production of enzymes by fungal strains using different agriculture wastes, no reports were documented on the production of amylase using sprout peel wastes as substrates.

With this background the present investigation entitled “Optimization of Amylase Production by *Aspergillus flavus* using agro waste as substrate” was undertaken with the following

Objectives:

- To determine the amylase activity on sprout peel waste as substrate.
- To study the production of amylase using *Aspergillus flavus*
- To optimize the fermentation conditions namely, incubation period, temperature and pH

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Safey and Ammar (2004), studied on purification and characteraization of α-Amylase isolation from Aspergillus flavus var. columnaris reported that the optimum substrate concentration (starch) was 0.2 % (w/v) while the optimum incubation temperature was 35ºC. The purified α-amylase enzyme had a maximum activity at pH 6.2, after 30 h of incubation.

Abu *et al*., (2005) investigated on Raw starch degrading amylase production by mixed culture of *Aspergillus niger* and Saccharomyces cerevisae grown on sorghum pomace and reported that the mixed culture media recorded higher ($P<0.05$) level of raw starch degrading amylase than monoculture media. Optimum pH for the raw starch degrading amylase which varied between 3.0 and 8.0 depended on the source of the crude enzyme.

Ertan *et al*. (2005) screened fungi for their ability to produce α-amylase by a plate culture method. *Penicillium chrysogenum* cultivated in liquid media containing maltose (2 per cent) reached its maximum activity hydrolyzed starches from different sources. The best hydrolysis was determined (98.69 per cent) in soluble starch for 15 minutes at 30 degree.

Banerjee and Negi (2006) studied the production of amylase and protease at their optimum level in a single bio reactor by modified solid state fermentation. Maximum yield of amylase and protease was achieved by using wheat bran as a substrate by a highly potent, locally isolated strain of *Aspergillus awamori*. Nakazawa MTCC 6652, strain has been induced previously, inferring the ability to produce both enzymes concomitantly in a single bioreactor with their maximum capacity.

Ertan and Balkan (2006) reported that solid state fermentation (SSF) was carried out using corn cob leaf (CL), rye straw (RS), wheat straw (WS) and wheat bran (WB) as substrates for α-amylase production by a fungal culture of *Penicillium chrysogenum*. WB showed the highest enzyme production with 160 U / ml under optimum conditions. The other enzyme activities were 28, 49 and 45 U / ml using CL, RS and WS respectively.

Kathiresan and Manivannan (2006) studied the effects of pH, temperature, incubation time, salinity, sources of carbon and nitrogen in submerged fermentation process in the production of α-amylase by *Penicillium fellutanum*. They used different carbon and nitrogen sources. Among these sources maltose was the best to enhance the enzyme activity of 146 U/ml and peptone was ideal to increase the enzyme activity of 150 U/ml. Alva *et al*., (2007) investigated on Production and characterization of fungal amylase enzyme isolated from Aspergillus sp. JGI 12 in solid state culture reported that the mixed culture media recorded higher ($P<0.05$) level of raw starch degrading amylase than monoculture media. Optimum pH for the raw starch degrading amylase which varied between 3.0 and 8.0 depended on the source of the crude enzyme.

Sun *et al*. (2007) studied the effect of the starch, protein and soluble oligosaccharide contents in wheat bran on the production of extracellular amylase by *Penicillium decumbens*. kwatia *et al*., 2007 studied on Optimization of amylase production by Aspergillus niger cultivated on yam peels in solid state fermentation using response surface methodology reported that The results of the study indicated that amylase is maximized (30.95 U/ml-min) at optimized levels of 49.53°C, 5.95 and 104 h for temperature, pH (initial) and incubation periods, respectively.

Tiwari *et al*. (2007) studied on α-amylase production with a fungal strain *Penicillium rugulosum* isolated from a soil sample. Among all carbon sources, galactose yield the highest production of extracellular amylase (2.23 $mgml^{-1}$) and peptone yield the maximum enzyme activity (2.41 $mgml^{-1}$) when compared with other nitrogen sources.

Yagar *et al.* (2008) investigated some properties of immobilized α-amylase by *Aspergillus sclerotiorum* within calcium alginate gel beads and compared with soluble enzyme. The percentage of soluble starch hydrolysis for soluble and immobilized α-amylase were determined to be 97.5 and 92.2 per cent for 60 minutes respectively.

Xu *et al*. (2008) reported that impressive amount of alpha amylase was produced from *Aspergillus oryzwe* with solid culture cultivation method. Solid state fermentation favored the production of amyloglucosidase significantly in cassava peels.

Diomi *et al*. (2008) assessed that the utilization of agro-industrial residues, including orange waste powder as the substrate for the fermentation. Jain *et al*. (2009) stated that *Penicillium chrysogenum* MTCC # 160 optimized the production of amylase when grown on starch containing basal salt medium exhibited the highest amylase production after 5 days of incubation. The optimum temperature and pH of amylases was recorded at 30°C and 5.0 – 7.5 respectively. He further studied on the activity of enzyme done with metal ions at 20 mM per ml of reaction mixture, Na^{+} and Ca^{++} ions gave more than 90 per cent relative activity whereas metal ions Cu^{++}, Cd^{+}, Hg^{++}, Mn^{++}, Li^{+} affected approximate 60 per cent activity. Phenol showed maximum inhibition (0.00 U/ml) followed by ammonium (1.5 U/ml) and acetic acid (4.5 U/ml) whereas aniline and benzene dose not affected and showed 95 per cent relative activity followed by ethyl alcohol and aldehyde (85 per cent).

Dhale and Raj (2009) isolated *Penicillium* sp. NIOM-02 from the marine sediment which produced red pigment. *Penicillium* sp. NIOM-02 grown on sugar cane baggase secreted more amylase (246 U/mg) in culture medium No. 5 and the zymogram analysis revealed its molecular mass (53 kDA).

Shafique *et al*. (2009) tested the number of substrates for cultivation of microorganisms to produce a host of enzymes. The effect of different substrates (wheat and rice straw, sugarcane waste, wood waste) and nitrogen sources (ammonium sulphate, urea, peptone, yeast extract, sodium nitrate) on growth and α-amylase activity was studied for the native and mutant strains. The most active synthesis occurred on wheat straw, at rates of 33.43, 71.42 and 86.38 U ml^{-1} for *A. niger* FCBP – 198, An – UV – 5.6 and An – CHh – 4.7 and among all nitrogen sources, sodium nitrate was choosen as the best for α-amylase production by all isolates with highest activity of 61.71, 99.59 and 110.94 U ml^{-1} for *A. niger* FCBP - 198, An – UV – 5.6 and An – Ch – 4.7.

Sindhu *et al*. (2009) studied the production of extracellular amylase by *Penicillium janthinellum* (NCIM 4960) under solid state fermentation using wheat bran as a solid substrate. The maximum enzyme activity obtained was 300 U / gds in the presence of maltose. Singh *et al*. (2009) studied α-amylase activity expression in *Humicola lanuginosa* under different environmental conditions using solid state fermentation on different agricultural byproducts. The α-amylase production was the highest at 155 hrs of incubation period, 50°C incubation temperature, 90 per cent initial moisture contents, pH of 6 and 20 per cent inoculum level and soluble starch was found the best supplementary carbon source.

Hashemi *et al*. (2010) studied the synergetic effect of low pH on activity of crude Ca-independent α-amylase isolated from a native *Bacillus* sp. KR-8104 in solid state fermentation (SSF) using wheat bran (WB) as a substrate. The effects of different parameters including moisturizing agents, solid substrate, moisture ratio, particle size, incubation temperature and period, inoculums (v/w) and supplementation with 1 per cent (w/w) different carbon and nitrogen sources on enzyme production were investigated. Maximum enzyme production of 140 U/g dry fermented substrate was obtained from wheat bran moistened with tap water at a ratio of 1 : 1.5 and supplemented with 1 per cent (w/w) NH(4) NO(3) and 1 per cent (w/w) lactose after 48 h incubation at 37 degrees.

Balkan and Ertan (2010) studied the production of new fungal amylase by solid state fermentation and purification of its biochemical properties. *Penicillium brevicompactum* showed the best enzymes activity according to screening methods with amylase degrading raw starch and *P. brevicompactum* was selected as an amylase source by wheat bran. The fermentation conditions were optimized for the production of amylase. The optimum fermentation conditions were found to be an initial moisture level for the solid substrate of 55 per cent, moistening agent of 0.1 M sodium phosphate buffer (pH 5.0), incubation period of 7-d-inoculum concentration of 2.5 ml and incubation temperature at 30°C.

Nouadri *et al*. (2010) reported that the amylase enzymes has a great significance due to its wide area of potential application. α-amylase from *Penicillium camemberti* PL 21, using orange waste as substrate produced under optimum conditions, after 168 h of incubation was subjected for purification and characterization. They observed that α-amylase activity increased by enzyme concentration rise. The purified enzymes exhibited 23.1 per cent of the total initial activity and there was a 38.5 fold increase in specific activity (154.2 units/ml/mg protein) when compared with the crude culture filtrate.

Nimkar *et al*. (2010) studied the production of α-amylase from *Bacillus subtilis* and *Aspergillus niger* using different agro waste by solid state fermentation. They found that the maximum enzyme activity was obtained in wheat bran (15.3 U / mg^{-1}) at 1.0 mg / ml concentration with *Aspergillus niger*.

Mishra and Dadhich (2010) were isolated fifteen strains of filamentous fungi from various regions of Rajasthan state. Among fifteen fungi he found out that the *Aspergillus Niger* RJ4 produced the highest extra cellular amylase activity (196.4 Ug^{-1}) followed by *Penicillium* sp. RJ6 (145.8 Ug^{-1}). Metin *et al*. (2010) observed that extracellular amylase produced by *Penicillium citrinum* HBF62 was purified 18 folds using starch affinity chromatography. The enzyme activity was inhibited in the presence of phenylmethanesulfonylfluride (PMSF). N-bromosuccinimide (NBS) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodimide methyl toluenesulphonate (CMC), suggesting that serine, tryptophan residues and carboxyl groups play an important role in the catalytic process. The activity of amylase was found to be tolerant upto 5 M NaCl concentration. Raw corn starch adsorption of amylase was found to be 74 per cent.

Taskin and Erdal (2010) investigated the feasibility of waste loquat kernels as substrate in solid-state fermentation for α-amylase production by *Penicillium expansum* MT-1. The fungus was isolated from fermented loquat kernels. Loquat kernel flour (LKF) could serve as a sole source of nitrogen and carbon for the fungus to grow and synthesize α-amylase. Additional carbon and nitrogen source increased the enzyme production. Supplementation of alcohols gave rise to a positive effect on the enzyme production.

Chinedu and Okochi (2011) studied on waste cellulosic materials (corncob, sawdust and sugarcane pulp) and crystalline cellulose induce 11 lulose production in wild strains of *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma harzianum* isolated from a wood-waste dump in Lagos, Nigeria. Cellulose-supplemented media gave the maximum cellulase activity of 0.54, 0.67 and 0.39 unit's mg protein-1 for *A. niger, P. chrysogenum* and *T. harzianum* respectively. The maximum enzyme activity for *A. niger* was obtained at 36 h of cultivation, while *P. chrysogenum* and *T. harzianum* gave their maximum enzyme activities at 12 and 60 h respectively. For the cellulosic wastes, highest enzyme activity was obtained with sawdust where *A. niger, P. chrysogernum* and *T. harzianum* gave the maximum enzyme activity of 0.30, 0.24, and 0.20 units mg protein-1 respectively after 144 hrs of cultivation. *A. niger* recorded the highest enzyme activity with any of the three cellulosic materials followed by *P. chrysogenum*. It thus appears that the use of sawdust presents the best option for low-cost commercial production of cellulose using *A. niger* and *P. chrysogenum*.

Sakthi *et al.* (2012) observed the evolution of amylase activity of the amylolytic fungi *Aspergillus niger* using cassava waste as a substrate and observed the maximum enzyme production of 4.12 U/ml was at 40o C and minimum of 3.12 U/ml was at 30oC. The maximum enzyme production 4.11 U/ ml was at pH 6 and minimum 3.11 U/ ml at pH 4.

Rajasekar *et al.* (2013) studied the production and optimization of amylases using Aspergillus niger and observed the optimum amylase activity of 334.51 µmol/ lit min in rice bran as substrate and 324.07 µ mol/lit.min at pH 6.0 on the fifth day of incubation. Oyewale (2013) in his study on statistical analyses of growth and amylase production of *Aspergillus flavus* on different carbon sources reported the best substrate for the growth and amylase production of 52. 60 mg/ml culture and 0.87 mg T.R.S released /mins /mg protein respectively.

Ruban *et al*. (2013) in their study on the production of amylase using starch waste as substrate by Bacillus subtilis and *Aspergillus niger*, reported that the highest amylase activity was at temperature range from 18 – 20 and the optimum temperature was attained at 20°C with a concentration of 9.70 mg/mol/min.

Zaferanloo *et al*., 2014, studied on the, amylase activity by Preussia minima, a fungus of endophytic origin studied using different carbon and nitrogen sources. They observed that enzyme activity was highest of 138 U/mg with starch as the carbon source and of value the optimum pH is 9 and optimum temperature was 25◦C.

Singh *et al*., (2014) studied on production of fungal amylase using cheap, readily available agriresidues, for potential application in textile industry. They observed the maximum enzyme activity at pH 6.0, temperature of 55◦C, and incubation time of 60 minutes. The study indicates that aspergillus *fumigatus* NTCC1222 can be an important source of amylase and the crude enzyme, hence obtained, can be cost effectively applied in multiple sections of textiles wet processing.

Veerapagu *et al*., (2016) studied on Screening and production of fungal amylase from *Aspergillus sp* by ssf. The results showed that the maximum enzyme production obtained with potato waste as substrate was 96 U/g followed by tomato (74 U/g) and brinjal and (58 U/g). Maximum enzyme production was found to be optimum at inoculums size 5% (78 U/g), incubation period 120 hr (98 U/g), pH of 5.0 (89 U/g) and temperature of 30ºC (86 U/g). Asrat and Girma 2018,studied on Isolation, production and characterization of amylase enzyme using the isolate *Aspergillus niger* FAB-211 and reported that the highest and least alpha-amylase production was found when 6 and 2 discs spore of *A. niger* FAB-211 were used, respectively. Maximum yield of alpha amylase (0.281 U/ml) was observed on the 3rd day of incubation period followed by 4, 6 and 5th days. Maltose and yeast extract were found to be the best carbon and nitrogen sources, respectively.

Ahmed *et al*.,2020 carried out a study on optimization and production of amylase by *Aspergillus terreus* using pomegranate peel waste and recorded the highest amylase activity of 340.69 U/ml using 1.5% pomegranate peel at 30◦C, pH 6.0 on 5 days incubation period .

MATERIALS AND METHOD

CHAPTER - III

MATERIALS METHOD

The present study is focused mainly on the isolation of efficient fungal strain capable of producing α –amylase enzyme and its optimization to conditions of pH, Temperature and incubation period and its protein estimation.

Collection of material

Palmyra sprout peel waste sample were collected from Palmyra sprout peel disposal area, kanapan nagar, Coimbatore district, Tamilnadu. The sample were taken and dry with a help of sun light and the dried sample were grinding with the help of mixes for the future study.

Growth medium for fungal culture

Sabouraud dextrose agar medium (SAD) – (Raymond sabouraud., 1892)- 250 ml

- Agar - 5g
- Peptone – 2.5 g
- Dextrose – 10 g
- PH - 5.6
- Streptomycin -1 ml
- Distilled water -250 ml

Combine all ingredient in 250 ml of distilled water and autoclaved at 120°c for 20 minutes and then cool to 45°C-50°C and pour into sterile petriplates and sterile test tubes for slant and also deep.

Inoculate fungal spores to the fungal media

After preparation of SDA media, now we are introducing the *Aspergillus flavus* spores into the pour SDA medium of petri plates and test tubes (as well as the agar deep and the agar slant) with a help of inoculation needle. This set up keep it in incubator at 34 °c for 72 hours.

Enzymology

Czapek-dox Liquid medium (Raper and Thom, 1949)

- Starch-10 g
- Sodium nitrate -2.0 g
- Potassium chloride -0.5 g
- Magnesium sulphate -0.5 g
- Dipotassium hydrogen phosphate -1.0 g
- Ferrous sulphate -0.01 g
- Distilled water -1000 ml
- Trace metal solution -1.0

Each liter of culture medium contains (999 ml) of the basal medium and the medium was supplemented with following trace element solution (1 ml).

Trace Metal Solution preparation

- Zinc sulphate -1.0 g
- Copper sulphate -0.5 g
- Distilled water -100 ml

PLATE – III

Process of Making Palmyra sprout peel waste Substrate

A – Dried peel of Palmyra sprout waste

B – Dried small piece of Palmyra sprout peel waste

C – Powder of Palmyra sprout peel waste

Preparation of culture media

100 ml of liquid medium was dispensed in 150 ml of cap bottle and sterilized at 1 atm for 15 minutes. After cooling, 1 ml of streptomycin sulphate (10000ppm) was added the pH of the medium was maintained at 6.0 and the isolated fungal strain of 5 g (disc) was inoculated and incubated for 5, 6, 7, and 4, 5, 6 days at 96, 120, and 144under static conditions. The experiment was carried out in triplicates for determining enzyme activity.

Preparation of culture filtrate as enzyme source (Extracellular)

The mycelium was filtered through Whatman no.40 filter paper using a Buchner funnel under suction and the clear filtrate was used as a source of extracellular enzyme.

Preparation of cell free enzyme source from mycelium (Intracellular)

The fungal mycelium was washed with distilled water twice a quantity of 5g of the washed mycelia mat was macerated in five ml of sodium phosphate buffer of PH 7.0 for α-amylase enzyme activity in a pre- chilled mortar and pestlewith a pinch of acid washed sand. The homogenate was centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 minutes. The supernatant served as an enzyme source.

Estimation of α-amylase enzyme activity (Miller, 1959)

Principle

Amylase activity was determined by the production of reducing groups from starch using DNS method.

Reagents

- Sodium phosphate buffer (0.1 m, PH -7.0)
- 1% of soluble starch
- Di-nitrosalicylic acid reagent (DNS): 1 g of dinitrosalicylic acid, 200 mg of crystalline phenol and 50 mg sodium sulphate were dissolved in 100 ml of 1% sodium hydroxide.

Procedure

- 0.5 ml of enzyme extract was pipette out in a test tube.
- 1 ml of 1% starch was added into the test tube.
- The mixture was incubated for 30 minutes at 370c.
- The content was cooled and 1 ml of DNS reagent was added and heated for 10 minutes.
- Final content was cooled with distilled water.
- The absorbance was measured at 540 nm.
- A standard graph was prepared with starch in the concentration range 50mg/ml

Calculation

The enzyme activity was expressed as the mg starch released min mg^{-1} protein.

Protein estimation (Lowry et *al.,* 1951)

Principle

The blue color developed by the reduction of phosphomolybdic phosphotungstic components in the folin ciocalteau reagent by the amino acid tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of protein with the alkaline cupric tartrate are measured in the Lowry's method.

Materials and method

- Reagent A – 4 g of sodium carbonate in 0.2 g of sodium hydroxide make up into 50 ml of distilled water.
- Reagent B - 0.5 g of copper sulphate and 1 g of potassium tartrate make up into 25 ml of distilled water.
- Reagent C – 50 ml of A and 1ml of B were mixed prior to use
- Reagent D – folin-ciocalteau reagent

Protein Solution (Stock Standard)

- 50 mg of bovine serum albumin (Fraction V) was weighed accurately and dissolved in distilled water and the volume was made up to 50 ml in a standard flask.

Working Standard

- 10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask (one ml of this solution contains 200 mg protein).

Procedure

- Extraction is usually carried out with buffers used for the enzyme assay.
- A quantity 500 mg of the sample was ground well with pestle and mortar in 5 ml of the buffer.
- The supernatant was centrifuged and used for protein estimation.

Estimation of protein

- 0.2, 0.4, 0.6, 0.8, and 1.0 ml of working standard was pipetted out in a series of the test tubes and 0.1 ml of the sample extract was pipetted out in the test tube.
- The volume was made up to 1 ml in all the test tubes. A tube with one ml of water served as the blank.
- 5 ml of reagent c was added to each test tube including the blank, mixed well and allowed to stand for 10 minutes.
- Then 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes. The blue color developed was read at 660 nm.
- The amount of protein in the sample was calculated by drawing a standard graph.

Calculation:

The amount of protein in the enzyme assay was expressed in $mg^{-1}g^{-1}$.

Optimization of culture conditions for enzyme production

The isolated fungal strain was inoculated in the nutrient medium (production medium) and was subjected to various pH, temperature, incubation period, with 0.1% palmyra sprout peel as substrate for the optimal production of the enzyme. All sets have been performed in triplicates and the statistical errors have been reported.

Effect of incubation period on enzyme production

The fungal strain isolated was inoculated in 100 ml of the production medium at static condition for a period of4th, 5th and 6th days of incubation at 30◦C, 35◦C, 40◦C. All sets have been performed in triplicates and enzyme activity was estimated.

Effect of temperature on enzyme production

The fungal strain was inoculated in 100 ml of production medium and incubated at

Varying temperature ranges (30C, 35C, 40C) under static condition. All sets have been performed in triplicates and the enzyme activity was estimated at5thday of incubation.

Effect of pH on enzyme production

The fungal strain was inoculated in 100 ml of production medium which was prominently adjusted by using 1 N HCl or 1 N NaOH to various pH ranges (5, 6, 7) and kept in static condition at 35°C. The experimental set up was done in triplicate sand the enzyme activity was estimated at 5thday of incubation.

Statistical Analysis

Standard errors of means of all the replicates of each variable were computed using Computer Software; Microsoft Excel Data regarding the Amylase activity on, (Palmyra sprout peel waste powder) substrate, pH, incubation period and temperature. They were statistically analyzed using 3 way analysis of variance (ANOVA) followed by LSD method to delineate mean differences (Panse and Sukhatme, 1978).

RESULTS AND DISCUSSION

CHAPTER–IV

RESULTS AND DISCUSSION

The result pertaining to the present investigation are discussed under the following:

Optimization of culture condition for enzyme production

α- Amylase Activity in the presence of substrate

a) Effect of incubation period on enzyme production

b) Effect of pH on enzyme production

c) Effect of temperature on enzyme production

Optimization of culture condition for free enzyme

α- Amylase activity in the presence of substrate

a) Effect of incubation period on enzyme production

The fungus *Aspergillus flavus* exhibited an increase in the enzyme activity on the 5^{th}day of incubation in 0.1 per cent concentration in the presence of palmyra sprout peel waste as substrate. Intracellular α-amylase activity was recorded as the highest activity in starch (4.87 ± 0.13) Umg^{-1} on 5th day when compared to extracellular α-amylase activity in starch (4.85± 0.08) Umg^{-1} on 5^{th}day. The minimum intracellular α- amylase enzyme activity was recorded in starch (1.55 ± 0.48) Umg^{-1}on 4^{th}dayand extracellular α- amylase enzyme activity was recorded the least in 1.68 ± 0.31 Umg^{-1} on 4th day of incubation.

Similar results were obtained by Veerapagu *et al*., (2016) The results who reported the maximum enzyme production obtained with inoculums size 5% (78 U/g) and incubation period of 120 hr (98 U/g). Similarly Ahmed *et al*., 2010 reported that *Aspergillus terreus* using pomegranate peel waste showed the highest amylase activity of 340.69 U/ml using 1.5% pomegranate peel at 5 days of incubation period. According to Asrat and Girma 2018, the maximum yield of alpha amylase (0.281 U/ml) was observed on the 3rd day of incubation period followed by 4, 6 and 5th days by *Aspergillus niger*

TABLE-I

Amylase activity (Umg^{-1} protein) of *Aspergillus flavus* on different incubation period at 0.1% Palmyra sprout peel waste as a substrate at 5th day of Incubation

Palmyra sprout peel as Substrate		DAYS		
		4	**5**	**6**
Intra cellular	**Control**	1.55 ± 0.48	1.72 ± 0.11	1.67 ± 0.09
	With substrate	3.76 ± 0.32	4.87 ± 0.13	4.10 ± 0.17
Extra cellular	**Control**	1.68 ± 0.31	2.95± 0.12	2.11 ± 0.12
	With substrate	3.58± 0.08	4.85± 0.08	3.40 ± 0.04
SED (p<0.05)		**0.594 0.003**	**0.770 0.055**	**0.562 0.046**

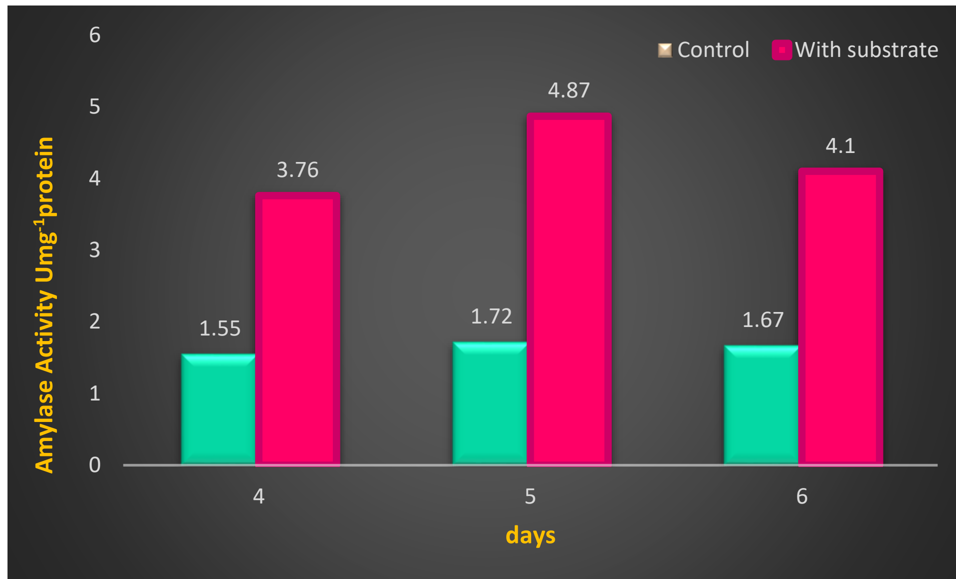
Results are the mean ± standard deviation of triplicates

Umg^{-1} = μmol starch released min^{-1} mg^{-1} protein

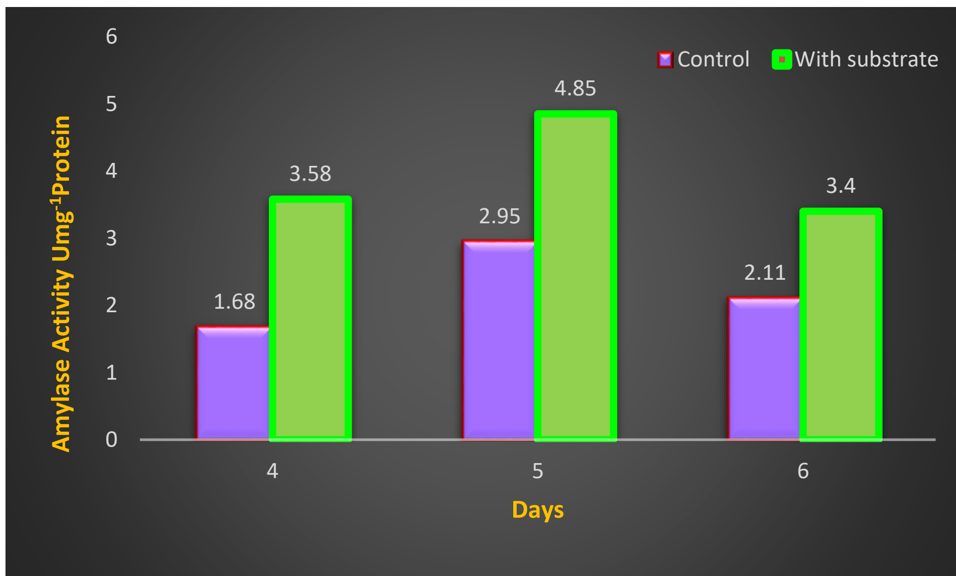
FIGURE –I

Amylase activity (Umg^{-1} protein) of *Aspergillus flavus* on different incubation period at 0.1% Palmyra sprout peel waste as a substrate at 5th day of Incubation

a. At An Intracellular level



b. At An Extracellular level



b) Effect of pH on enzyme production

An increase in the production of amylase was observed between the pH ranges 5.0 and 7.0 in Palmyra sprout peel. The amylase activity in 0.1% substrate at extracellular level showed an enhanced activity from 2.06±0.15to 4.93±0.07Umg^{-1}protein production up to a pH of 6.0 and after that its activity decreased (3.38±0.19Umg^{-1}protein) at a pH of 7.0 by *A.flavus* on the 5th day of incubation when compared to the intracellular level.

It may be observed from the statistical investigation that the pH enhanced the alpha-amylase production at an intracellular level ($P < 0.05 = 0.165$) at pH 6.0.

The results were on par with Asrat and Girma (2018) who observed the maximum activity of 0.483 U/ml at pH of 6.0.Similar results coincides the work of Singh *et al*., (2014). The maximum amylase enzyme activity was recorded in pH 6.0 by *Aspergillus fumigatus*. Our results are similar to Ahmed *et al*., (2010) they observed maximum amylase activity of 340.69 U/ml by *Aspergillus sp*. at pH 6.0.

TABLE II

Amylase activity (Umg^{-1} protein) of *Aspergillus flavus* on different pH at 0.1% Palmyra sprout peel waste as a substrate at 5th day of Incubation at 35°C

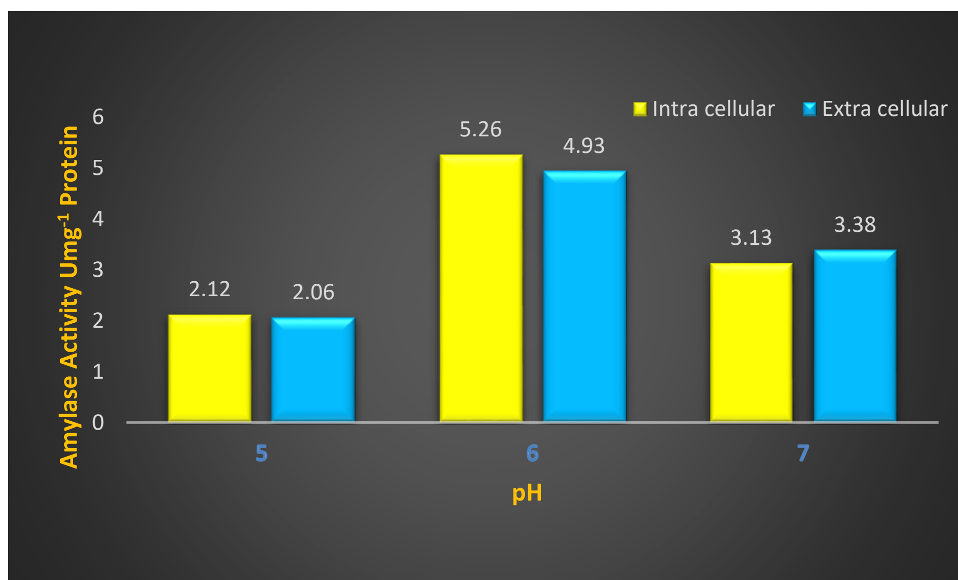
Palmyra sprout peel as Substrate	pH		
	5	**6**	**7**
Intra cellular	2.12	5.26	3.13
Extra cellular	2.06	4.93	3.38
SEd	**0.03**	**0.165**	**0.125**
(p<0.05)	**0.001**	**0.003**	**0.002**

Results are the mean ± standard deviation of triplicates

Umg^{-1} = µmol starch released min^{-1} mg^{-1} protein

FIGURE-II

Amylase activity (Umg^{-1} protein) of *Aspergillus flavus* on different pH at 0.1% Palmyra sprout peel waste as a substrate at 5th day of Incubation at 35°C



c) Effect of temperature on enzyme production

Temperature plays an important role in the enzyme production. The enzyme activity at both extracellular and intracellular level by Aspergillus flavus at different temperature ranges was found to increase from 30◦C to 35◦C and peaked at 35◦C and further increase in the temperature showed a decreasing trend.

The enzyme production was found to be significant in α-amylase production of5.96±0.10Umg-1protein in the intracellular level when compared to extracellular level of4.20±0.22 Umg-1 protein at 35◦C by *Aspergillus flavus* on 5th day of incubation.

Statistical analysis of data revealed that α-amylase activity was found to the activity at 35◦C (P<0.05= 0.880)

Table III

Amylase activity (Umg^{-1} protein) of *Aspergillus flavus* on different Temperature at 0.1% Palmyra sprout peel waste as a substrate at 5th day of Incubation at pH 6.0

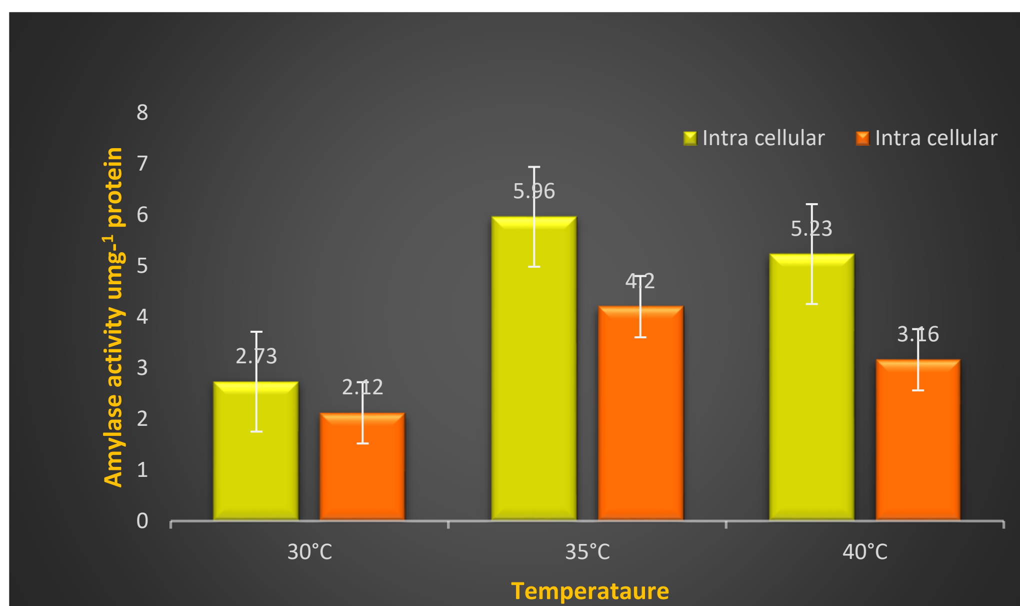
Palmyra sprout peel as Substrate	TEMPERATURE		
	30°C	35°C	40°C
Intra cellular	2.73±0.08	5.96±0.10	5.23±0.024
Extracellular	2.12±0.12	4.20±0.22	3.16±0.21
SEd (p<0.05)	**0.305 0.003**	**0.88 0.02**	**0.925 0.013**

Results are the mean ± standard deviation of triplicates

Umg^{-1} = µmol starch released min^{-1} mg^{-1} protein

FIGURE-III

Amylase activity (Umg^{-1} protein) of *Aspergillus flavus* on different Temperature at 0.1% Palmyra sprout peel waste as a substrate at 5th day of Incubation at pH 6.0



Similar results were observed by Ramachandran *et al.,* (2004) who reported that among the fungi, most amylase production studies have been conducted with mesophilic fungi within the temperature range of 25-37◦ C. The results were in accordance with the results of Sakthi *et al.,* (2012) observed the evolution of amylase activity of the amylolytic fungi *Aspergillus niger* using cassava waste as a substrate and observed the maximum enzyme production of 4.12 U/ml was at 40o C and minimum of 3.12 U/ml was at 30oC. (Ahmed *et al*.,2010).Singh *et al*., (2014) reported that the maximum enzyme activity at, temperature of 35-55◦.C, which is similar to our result.

SUMMARY AND CONCLUSION

CHAPTER–V

SUMMARY AND CONCLUSION

Starch belongs to the most abundant carbohydrate polymers on earth. It consists of glucose monomer units linked up by the α-glucosidic linkages. The starch consists of two district fractions amylase-linear α-1, 4-linked glucans, and amylopectin-linear α-1, 4-linked glucans branched with α-1,6 linkages, the enzymes responsible for its hydrolysis are called amylolytic enzymes. Three most known amylolytic enzymes are α-amylase, β-amylase and glucoamylase. Amylases are ubiquitous in distribution with plants, bacteria and fungi being the predominant source.

α- amylase may be derived from several bacteria, yeasts and fungi. Bacterial amylase however, is generally reefed over fungal amylase due to several characteristic advantages. Strain of *Aspergillus sp*. and *Bacillus sp*. mainly *Bacillus* amyloliquefaciens and *B. licheniformis* are employed for commercial applications. Enzymes are currently used in several different industrial products and processes and there is an ever increasing demand to replace traditional chemical processes with advanced biotechnological processes involving microorganisms and enzymes (Adeleke *et al.,* 2012).

The present research was undertaken to determine the optimum conditions and parameters for enhancing the amylase enzyme production by *Aspergillus flavus* in concentrations of palmyra sprout peel waste as substrates and also to assess its stability at different pH and temperature on 5th, 6th and 7^{th}day of incubation.

To achieve this goal, an in vitro investigation was carried out to assess the enzyme production by *Aspergillus flavus* isolated from palmyra sprout peel waste.

The results of the investigation are summarized below:

1. Intracellular α-amylase activity was recorded as the highest activity in starch when compared to extracellular α-amylase activity on 5th day.
2. The enzyme production was found to be significant in α-amylase production in the intracellular level when compared to extracellular level at 35◦C by *Aspergillus flavus* on 5th day of incubation.
3. The amylase activity of 0.1% substrate at extracellular level showed an enhanced activity at a pH of 6.0 on the 5th day of incubation at 35◦C

Conclusion

In most of the countries where the economy is largely based on agriculture and farming practice is very intensive, accumulation of agricultural residues is a serious problem. Utilization of agricultural residues through biotechnology is becoming more and more significant with the dual goal of waste disposal and value addition. Since microbial activity especially fungal activity is the key aspect in this area, there is enormous opportunity for the cost effective production of amylase.

Thus, it can be deduced from the present investigation that the candidate *Aspergillus flavus* can be effectively harnessed for the maximum production of amylase enzyme and the use of waste materials as substrates for the enzymes production is eco-friendly and a promising step to solve issues of environmental pollution.

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