Production of α-amylase by solid state fermentation of apple pomace

Dissertation submitted in partial fulfillment of the requirement for the degree of

Bachelors of Technology

In

Biotechnology

By

AMIT BHANDARI

UNDER THE GUIDANCE OF

Dr. Anil Kant

JAYPEE UNIVRSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

MAY 2018

Table of Contents

TOPIC	Page No.
DECLERATION BY SCHOLAR	**IV**
SUPERVISOR'S CERTIFICATE	**V**
ACKNOWLEDGEMENT	**VI**
LIST OF ACRONYMS AND ABBREVIATIONS	**VII**
LIST OF FIGURES	**VIII**
LIST OF TABLES	**X**
ABSTRACT	**XI**
1) CHAPTER 1- Introduction	**2**
2) CHAPTER 2- Review of Literature	**5**
2.1 Composition of apple pomace	6
2.2 Solid state fermentation involving apple pomace	7
2.2.1 Ethanol production	7
2.2.2 Enzyme production	7
2.2.3 Organic acid production	8
2.2.4 Nutritionally enriched feed	9
2.2.5 Pigment production	10
2.2.6 Other products	10
2.3 α-amylase production by solid state fermentation	11
2.3.1 Bacteria used in SSF for α-amylase production	11
2.3.2 Fungi used in solid SSF for α-amylase production	11

3) CHAPTER 3- Materials and methods 13

3.1 Microorganism used 13

3.2 Culture revival and inoculums preparation 13

3.3 Staining of fungal culture 13

3.4 Reagent used 13

3.4.1 Salt Solution 13
3.4.2 Tween-80 Solution 14
3.4.3 1% soluble starch 14
3.4.4 0.1% Acetate buffer 14
3.4.5 DNS (Di-nitro Salicylic acid) 14
3.4.6 Perchloric acid 14
3.4.7 Anthrone 14

3.5 Quantification of starch content in apple pomace 14
3.6 Quantification of reducing sugar in fermented apple pomace 15
3.7 Enzyme assay of α-amylase 15
3.8 Statistical analysis 15
3.9 Methods to set up SSF in various experiments 15
3.10 Optimization of initial moisture content. 17
3.11 Optimization of fermentation time 18
3.12 Optimization of pH of substrate 18
3.13 Optimization of nitrogen source 18
3.14 Optimization of different substrate combination with apple pomace (1:1) 19
3.15 Optimization of different ratio of potato peel with apple pomace
3.16 Optimization of different ratio of wheat bran with apple pomace
3.17 Observation recorded 20

3.14.1 Fungal culture 20
3.114.2 Spore Count 20

4) CHAPTER 4- Results and Discussion 21

4.1 Starch Content in apple pomace 21

4.2 Optimization of moisture content 21

4.3 Optimization of fermentation time 22

4.4 Optimization of pH 23

4.5 Optimization of Nitrogen source 24

4.6 Combinations of various substrates (1:1) with apple pomace 25

4.7 Various combinations of potato peels with apple pomace. 26

4.8 Various combinations of wheat bran with apple pomace. 27

5) Chapter 5- Conclusion 36

5) References 37

DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the B-Tech thesis entitled **"Production of α-amylase by solid state fermentation of apple pomace"** submitted at **Jaypee University of Information Technology,Waknaghat India,** is an authentic record of my work carried out under the supervision of **Dr. Anil Kant**. I have not submitted this work elsewhere for any other degree or diploma.

Amit Bhandari

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat, India

Date:

SUPERVISOR'S CERTIFICATE

This is to certify that the work titled "**Production of α-amylase by solid state fermentation of apple pomace**", submitted by **Amit Bhandari** for end semester (Even) of Bachelor of Technology in Biotechnology Engineering to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision. This work has not been submitted partially or fully to any other university or Institute for the award of this or any other degree or diploma.

Signature of Supervisor:

Name of Supervisor Dr. Anil Kant

Designation Associate Professor

Date:

ACKNOWLEDGEMENT

Known words become inadequate to express the gratitude for my mentor and guide Dr. Anil Kant, Associate professor in Department of Biotechnology and Bioinformatics, who initiated me into the realm of research and supervised me with finite patience and without whose invaluable suggestion and unstinted co-operation, the present desertion would not have been possible. I'm also thankful to Lab staff of Biotechnology department for their continuous support and encouragement. Also, I would like to thank the officials of Jaypee University of Information technology (JUIT), Waknaghat for their help and cooperation.

Signature of student:

Name of Student Amit Bhandari

B.tech (Biotechnology)

Date:

LIST OF ACRONYMS AND ABBREVIATIONS

SSF	Solid State fermentation
SmF	Submerged Fermentation
U/gds	Units per gram dry substrate
U/ml	Units per Millie liter
DPPH	2,2-diphenyl-1-picrylhydrazy
˚C	Degree centigrade
gm	Gram
mg	Millie gram
PDA	Potato dextrose agar
DNS	Di-nitro salicylic acid
nm	Nanometer
r.p.m	Round per minute
min	Minute
hrs	Hours
M	Molar
AP	Apple pomace
PP	Potato peel
WB	Wheat Bran
O.D	Optical Density

LIST OF FIGURES

Figure No.	Title
2.1	Flowchart for apple processing for juice production
3.1	Apple pomace before sieving and crushing
3.2	Apple pomace before sieving and crushing
3.3	Apple pomace after crushing and seiving
3.4	Spore viability test to check the viability and re-growth of spores on PDA media.
3.5	Fungus observed under 40X magnification after LCB staining.
4.1	Enzyme activity of α-amylase obtained at different moisture ratio of Apple pomace by *Aspergillus oryzae.*
4.2	Mean enzyme activity of α-amylase depicting the best moisture ratio (70%) for enzyme production.
4.3	Enzyme activity of α-amylase obtained after different fermentation time of Apple pomace by *Aspergillus oryzae.*
4.4	Mean enzyme activity of α-amylase showing the best fermentation time (96hrs) of apple pomace by *Aspergillus oryzae*
4.5	Enzyme activity of α-amylase in triplicates obtained after different pH after fermentation of Apple pomace by *Aspergillus oryzae.*

4.6	Mean enzyme activity of α-amylase showing the best pH for the fermentation of apple pomace by *Aspergillus oryzae.*
4.7	Enzyme activity of α-amylase in triplicates obtained after supplementation of different nitrogen sources (0.25M) with apple pomace and fermentation by *Aspergillus oryzae.*
4.8	Mean enzyme activity of α-amylase showing the best pH for the fermentation of apple pomace by *Aspergillus oryzae* (1- Ammonium sulphate, 2- Ammonium Nitrate, 3- Ammonium phosphate, 4- Sodium Nitrate, 5- Ammonium chloride).
4.9	Enzyme activity of α-amylase in triplicates when different substrates are mixed with apple pomace at 1:1 ratio.
4.10	Mean enzyme activity of α-amylase showing different mixtures of substrate with apple pomace at 1:1 ratio after fermentation by *Aspergillus oryzae* (1- Mustard oil cake: Apple pomace (1:1), 2- Rice husks: Apple pomace (1:1), 3- Potato peels: Apple pomace (1:1), 4- Wheat bran: Apple pomace (1:1).
4.11	Enzyme activity of α-amylase obtained after different ratios of potato peels with apple pomace (1- potato peel: apple pomace (1:1), 2- Potato peel: apple pomace (1:2), 3- Potato peel: apple pomace (1:3)) after fermentation of Apple pomace by *Aspergillus oryzae.*
4.12	Mean enzyme activity of α-amylase showing different ratio of potato peels with apple pomace after fermentation by *Aspergillus oryzae* (1- Potato peel: apple pomace (1:1), 2- Potato peel: apple pomace (1:2), 3- Potato peel: apple pomace (1:3)).
4.13	Enzyme activity of α-amylase obtained after different ratios of wheat bran with apple pomace (1- Wheat bran: apple pomace (1:1), 2- Wheat bran: apple pomace (1:2), 3- Wheat bran: apple pomace (1:3)) after fermentation of Apple pomace by *Aspergillus oryzae.*
4.14	Mean enzyme activity of α-amylase showing different ratio of wheat bran with apple pomace after fermentation by *Aspergillus oryzae* (1- Wheat bran: apple pomace (1:1), 2- Wheat bran: apple pomace (1:2), 3- Wheat bran: apple pomace (1:3)).

LIST OF TABLES

Table No.	Topic	Page No.
2.1	Apple pomace composition	6
4.1	Various samples of apple pomace analyzed for starch content	21
4.2	Mean enzyme activity of α-amylase at different moisture content in apple pomace after SSF by *Aspergillus oryzae.*	22
4.3	Mean enzyme activity of α-amylase at different fermentation time obtained under SSF of apple pomace by *Aspergillus oryzae.*	23
4.4	Mean enzyme activity of α-amylase at different pH of apple pomace under SSF by *Aspergillu oryzae*.	24
4.5	Mean enzyme activity of α-amylase at different nitrogen sources supplementation obtained by SSF of apple pomace by *Aspergillus oyzae.*	25
4.6	Mean enzyme activity of α-amylase obtained after SSF of apple pomace supplemented with other substrates in 1:1 ratio.	26
4.7	Mean enzyme activity of α-amylase at various ratios of Potato peel with apple pomace obtained under SSF of apple pomace by *Aspergillus oyzae.*	27
4.8	Mean enzyme activity of α-amylase showing different ratio of wheat bran with apple pomace after SSF by *Aspergillus oryzae*	27
4.9	Effect of different parameter (Fermentation time, Moisture content, pH and various substrate ratios) on the activity of α-amylase produced by *Aspergillus oryzae* by SSF of Apple pomace.	35

ABSTRACT

Alpha -amylase along with other enzymes makes 44% of total share of Indian enzyme market and alone forms 25% of world's enzyme market. Amylases produced by fungal and bacterial sources have wide commercial application in various industries like Food, Pharmaceutical, Detergent, Paper and pulp and Fuel-alcohol producing industries. Apple pomace is an underutilized waste in juice and apple product industries and it posses disposal problem. Out of 1 million tons per annum production of apple pomace only 10,000 Kg is utilized, and majorly it's been used as cattle feed. Apple pomace is rich in carbohydrates, free nitrogen extract and proteins. Solid state fermentation (SSF) increases the amount of protein in the waste which makes it a better feed for the cattle after the fermentation. Utilization of apple pomace for the production of high value enzymes would be more valuable to Himachal Pradesh where there is no dearth of raw material and it would be easy to set up small scale industry. This study was focused on the production of α-amylase employing solid state fermentation by *Aspergillus oryzae* on apple pomace and mixture of various agro-industrial wastes. Experiments were designed to optimize different fermentation parameter like initial moisture ratio, fermentation period, pH, nitrogen sources, and various substrate mixtures with apple pomace. The optimum fermentation conditions for the production of enzyme were 70% of initial moisture content, 96 hrs of fermentation period, pH 5 and sodium nitrate as nitrogen source at 0.25M. Among various agro-industrial wastes wheat bran and potato peels showed comparable amount of enzyme production, these substrates were further evaluated at different ratios with apple pomace to get maximum enzyme titer. Wheat bran showed highest enzyme titer when mixed in ratio of 1:1 with apple pomace and potato peels showed maximum enzyme titer at ratio 1:2.

CHAPTER-1
Introduction

Apple pomace is the leftover residue disposed from the juice industries containing seeds, peels and solid part which comprises around 25-35% of the fresh apple used for juice extraction. Apple pomace faces considerable disposable problem with mostly being dumped in open ground which causes serious environmental and public health problem like foul smell, microbial growth leading to potential health hazard. Since apple pomace is high in moisture content that makes it bulky and heavy leading to increase in the cost of transportation hence preventing proper disposition by the juice factories.

Out of 1 million tones Kg of apple pomace produced in India only 10,000 tons is utilized, most primarily as animal feed. This application of apple pomace is also limited as it is not rich in protein and vitamins which means low nutritional values.

Apple pomace is highly biodegradable and its wastage is the potential loss of biomass that could be used for producing commercially valued products. Lots of researches have been carried out to produce value added products using apple pomace. Apple pomace has been utilized using both SSF and SmF fermentation for the production of value added substances and it has shown its potential to be good substrate for solid state fermentation.

Alpha-amylases are the enzymes produced extracellularly that randomly cleaves the 1,4-α-D-glycosidic bonds between adjacent glucose molecules in a linear amylose chain generating units of glucose, maltose and maltotriose. Alpha amylases are found in plants, animals and microorganisms, majorly microbial enzymes are used in industries. Microbial alpha-amylases have almost completely reduced the dependency on chemical processes used for hydrolysis of the starch. Alpha amylases are nether most commercially valued enzymes having their application widened from starch saccharification to textile, baking, brewing, paper and distillation industry. Fungal and bacterial amylases have found large industrial applications with both of them having certain advantages over the other. Most widely used bacterial alpha-amylases are

derived from *Bacillus* species, they hydrolyze starch in the starch liquefaction process and converts starch in glucose and fructose syrups. Fungal amylases have more proficiency in starch saccharification when compared to bacterial amylases. Amylase produced from *Aspergillus* species finds numerous applications in baking industry (antistaling), clarification of fruit juices and alcoholic beverages.

SSF is defined as the fermentation using solid matrix as the substrate which is been carried out in modest amount of free water; however, the substrate should have adequate nutrition and moisture content to support the growth and metabolism of the growing microbes. In past few years solid state fermentation has gained lots of interest because of its potential to be used as the alternative for the conglomeration of bioprocesses like biopulping, bioleaching, bioremediation, biobenefications, biotransformation, biological detoxification etc. SSF has also been used to produce many value added products including enzymes, organic acids, biopesticides, vitamins, pigments, Biosurfactants, Ethanol, Aroma compounds (Pandey et al., 2000).

Advantages of Solid state fermentation in comparison to its counterpart submerged fermentation-

1. The increasing trend of SSF in biotechnology is because of the simplicity and cost-effective way of producing high titers of products than that in submerged fermentation (SmF).
2. Less need of maintaining sterility, as the moisture content in the substrate is limited so only the desired microbe tends to grow and it eliminates other contaminating bacteria.
3. Cost effective process, SSF is generally employed using agro-industrial wastes or cheap substrates and the process run on low-energy consumption due to less need for agitation which reduces the cost of the fermentation process.
4. Usage of simple media and less requirement of reagents reduces foaming which is a big problem in SmF.
5. Fungus grows better in SSF as it mimics the natural habitat of the fungus i.e low moisture conditions.

Agro-industrial wastes are substantially used as the substrate for solid state fermentation as they prove to be suitable substrate. They not only provide support for the anchorage of cells but provide nutrition to the growing microbial culture, along with other factors like cost–effectiveness and availability which makes them good substrate for SSF.

There are various factors (physiological and biochemical) which play important role in growth and activity of the microorganism over a substrate in SSF which includes particle size, moisture content, pH, temperature of incubation, age and size of inoculum, supplementation of additional nutrients like N, P and trace elements, additional requirement of carbon and nitrogen sources, addition of inducers. The relevant process factors need to be identified and optimized for a particular substrate.

SSF encompasses lots of advantages, disadvantages, and challenges which need to be considered and worked to make this technology more feasible to use. In spite of various advantages there are various challenges which surround SSF. Two major challenges are limited mass and heat transfer in large-scale production and scaling up of SSF. Solids have very low thermal conductivity coupled with large amount of heat generation by microbes which sometime leads to inactivation of product. Scale-up problems are also encountered during SSF, as all the parameters considered in the laboratory scale are difficult to emulate at the industrial scale. Biomass estimation also becomes a challenge in solid state fermentation when dealing with fungus as its mycelium forms interaction between solid substrate which makes the complete quantitative estimation of biomass very difficult. Difficulty in establishing kinetics of the reaction in SSF is also a challenge. All these challenges along with some other factors make the solid state fermentation still underdeveloped method to be completely accepted and used in industries. There are various objectives which are focused in this study-

Objective of the research

1. To establish sustainable production of alpha-amylase by SSF of apple pomace.
2. To optimize the various parameters which are needed for the growth and production of the enzyme by *Aspergillus oryzae*.
3. To optimize the best ratio of other agro-industrial wastes with apple pomace to get the maximum production of alpha-amylase.

CHAPTER 2

Review of Literature

India produces one third of the total apple produced around the world, out of which around 30% are used for the preparation of industrial product like jams, jellies, cheddar, wine etc and out of the 30% majority of apples are used for juice preparation. The residue left over after juice extraction (25%-30%) is known as "Apple pomace" which constitutes for about 1 million tons (Shalini et al., 2010). Flow chart in the figure 2.1 shows the utilization of apples in industries. There are many researches which have taken place to test apple pomace as a substrate for the production of value added products. Various product produced using apple pomace as substrate are enzymes (Berovič et al., 1997, Sun et al., 2010, Joshi et al., 2006), organic acids (Stredansky et al., 2000, Hang et al., 1986 Gullon et al., 2008), nutritionally enriched feed (Joshi et al., 1996, Villas et al., 2003, Vendruscolo et al., 2009, Rahmat et al., 1995), ethanol (Hang et al., 1982, Sandhu et al., 1997), pigment production (Attri et al., 2005, Joshi et al., 2011), aroma compounds (Almosnino et al., 1996), biogas (Kalia et al., 1992) and many more.

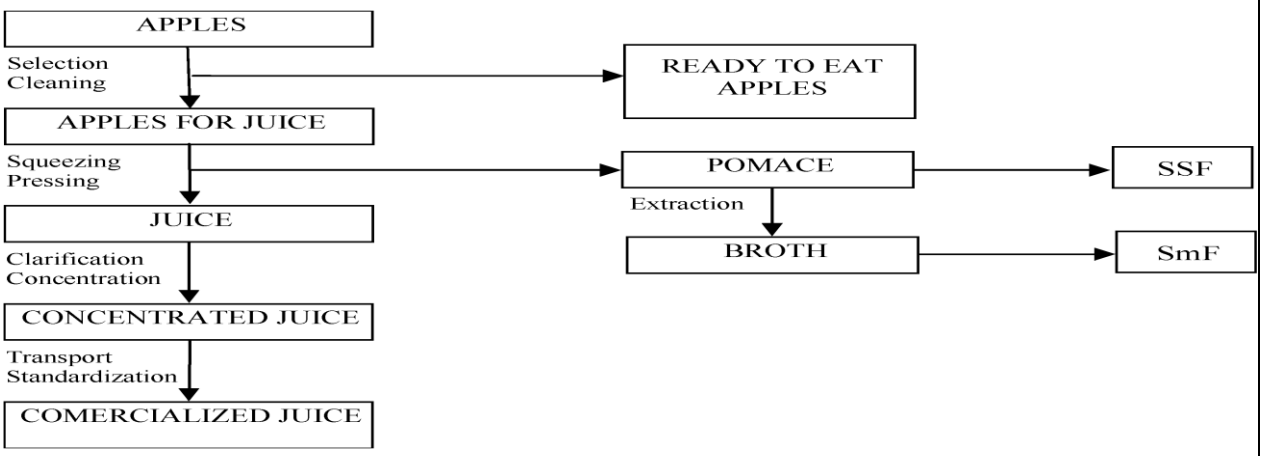

Figure 2.1 Flowchart depicting the processing of apple in juice industries

2.1 Composition of apple pomace

Apple pomace is high in moisture content and is mainly composed of insoluble carbohydrates such as cellulose, lignin and hemicelluloses. It also contains simple sugar such as glucose, fructose and sucrose with small amount of minerals and proteins. Table 2.1 shows the composition of apple pomace as quantified by Albuquerque et al., (2003). Apple pomace is also a rich source of pectic substances and is being used as the source for pectin throughout the world. Sharma et. al., (2014) developed the process for pectin extraction from apple pomace by treating with 0.05M HCL for an hour and then precipitation using 95% ethanol. The yield was 10.5% on dry weight basis and the quality obtained was comparable to other methods. The cost of extraction of pectin was found out to be Rs. 808.30 per Kg.

Table 2.1- Apple pomace composition (Albuquerque et al., 2003)

Composition	**Percentage (Wet weight Basis)**
Moisture	79.2%
Protein	3.7%
Lipids	n.d
Fibers	38.2%
Ashe	3.5%
Carbohydrates	59.8%
Reducing Sugar	10.8%
Pectin	7.7%
pH	4.0%
Titratable activity	0.13%
Water activity	0.973%

Apple pomace also has significant amount of dietary fibers which are non-starch polysaccharides with good amount of insoluble fibers (36%) as well as soluble fibers (14%). Use of apple pomace as the source of dietary fibers in various food articles has also been studied. Masoodi et al., (2002) observed the cake quality when apple pomace of different particle size and different concentration was mixed in wheat flour for cake making. The pH and specific gravity of the batter decreased with the increase in the

concentration of pomace whereas cake weight, shrinkage and uniformity index increased. Investigations were also done on using apple pomace as the potential source for polyphenols (Lu et al., 1997). Lu et al., (2000) examined apple pomace polyphenols using β-carotene/linoleic acid system, DPPH assay and superoxide scavenging activity. DPPH radical scavenging activity was 2-3 times and super-oxide anion radical scavenging activities were 10-20 times more than those of antioxidants vitamin C and E.

2.2 Solid state fermentation involving Apple pomace

Apple pomace has previously been used as a substrate in solid state fermentation for the production of value added products. For this purpose various microbes (fungi and bacteria) have been cultivated on apple pomace. Majorly filamentous fungi are very suitable organism to grow over organic residue (Apple pomace) because of their ability to infest over the surface and also inside the solid medium. Some industrially important product produced by solid state fermentation on apple pomace are-

2.2.1 Ethanol production

Apple pomace has been subjected to solid state fermentation by *Saccharomyces cerevisiae, Candida* and *Torula* for the production ethanol (Sandhu et al., 1997). Around 89% of fermentation efficiency was achieved for the production of ethanol for apple pomace using *Saccharomyces cerevisiae* Montrachet strain (Hang, et al., 1981). In solid state fermentation system Chatanta, et al., (2008) observed increase in ethanol production 16.09% (v/w) when SSF was done using co-cultures of *S. cerevisiae* MTCC 173, *A. Foetidus* MTCC 151, *F. oxysporum* MTCC 1755 when compared to 8.44% (v/w) by single strain of *S. cerevisiae* MTCC 173 after 72 hrs of incubation at 30° C.

2.2.2 Enzyme production

One of the most interested areas for the utilization of apple pomace in solid state fermentation is by its use as a substrate for enzyme production. Various enzymes like Pectinase, xylanase, cellulase, β-glucosidase have been reported to be produced using apple pomace. Sun et al., (2010) optimized the conditions for the production of cellulase under SSF of apple pomace using *Tricoderma* species. Incubation temperature, inoculum size and initial moisture of the substrate were critical conditions in the

production of enzyme. The optimum conditions were initial moisture level (70%), incoulum size ($2x10^8$spores/ml) and incubation temperature (32° C). The addition of the inducer lactose and nitrogen source corn-steep solid to the apple pomace improved the enzyme production reaching 7.6 U/gds.

Berovič (1997) produced pectolytic enzymes by solid state fermentation of apple pomace using *Aspergillus niger*. The parameters that were optimized in laboratory scale were also tested in a 15 litre horizontal stirred tank reactor. Effect of various parameters like inoculation volume, aeration, mixing, temperature and moisture were studied on the production of pectolytic enzymes. Various other agro-industrial wastes were mixed with apple pomace like wheat bran, soya flour and simple mineral salt. The best enzyme activity of 320U/ml was found at 38% moisture content at 35° C with high concentration of inoculums ($5x10^8$conidia/ml).

Joshi et al., (2006) produced Pectin methyesterase from *Aspergillus niger* by solid state fermentation of apple pomace at pH 4, temperature 25° C for 96 hrs and compared the enzyme activity. Overall Pectin methyesterase activity was found 2.3 times higher in SSF than SmF, after optimizing all the fermentation parameters.

Apple pomace was also used as the substrate for the production of β-glucosidase employing SSF from three *Aspergillus* species with *Aspergillus foetidus NRH377* showing the best activity. The enzyme was partially purified and the optimum activity was found at 65°C and pH 4.6.

Dhillon et al., (2012) optimized the SSF process parameters for the production of xylanase and also demonstrated the scale up capacity in solid state tray fermentor. At laboratory scale the best xylanase production (3578 IU/gds) was found at 85% initial moisture, pH 5, Inducers (Veratryl alcohol 2mM/Kg, Lactose 2%(w/w), Copper sulphate (1.5mM/Kg) after 48 hours. The xylanase activity was found to be 3952 IU/gds after 72hrs in solid state tray fermentation.

2.2.3 Organic acid production

Hang et al., (1986) demonstrated the production of citric acid by solid state fermentation of apple pomace by *Aspergillus niger* NRRL567, the study showed that the amount of methanol content in pomace, fermentation time and temperature are the key factors affecting the production of enzyme. The yield was 90gm/kg of pomace. Using the same

strain Dhillon et al., (2011) produced citric acid on apple pomace supplemented with rick husk, they studied the effect of two parameters like moisture content and effect of inducers. The best production was found to be (342·41 g/kg and 248·42 g/kg dry substrate) using two inducers ethanol and methanol (3% each) at 75% (v/m) moisture in 144hrs. In tray fermentation the best concentration of citric acid was found out to be 187.96 g/kg and 303.34 g/kg with ethanol and methanol (3%) respectively. Apple pomace is also been used to produce γ-Linolenic acid from *Thamnidium elegans* (Stredansky et al., 2000).

2.2.4 Nutritionally enriched feed

Primarily apple pomace has always been used as animal feed but because of its low nutrient proportion (particularly proteins) and easily perishable nature (due to high moisture content) its use is limited.

Joshi et al., (1996) concomitantly produced ethanol and animal feed from apple pomace by drying the pomace left after ethanol removal. Three different yeasts were used for the fermentation. After the fermentation, dried pomace was found to be rich in crude protein (3 times), fat (1.5times), and vitamin C (2 times) along with increase in the mineral, crude fibers and ash content, when compared to unfermented pomace.

Villas et al., (2003) treated apple pomace with two fungus *Candida utilis* and *Pleurotus ostreatus* individually and sequentially for bioconversion of pomace into enriched substrate and better digestibility for ruminant feed. The pomace sample treated with *Candida utilis* showed 100% protein increment, 60% mineral increment and 8.2% increased digestibility. When both the fungus were used the protein content increased by 500% after 60 days of fermentation with good increase in mineral levels. The amount of free sugar decreased after mixed fermentation and alone with *Candida utilis* whereas the free sugar increased after *P.ostreatus* fermentation.

Protein enrichment of apple pomace to be used as the feed for fish was also investigated. Vendruscolo et al., (2009) used *Gongronella butleri* for solid state fermentation of apple pomace. Various parameters were optimized like nitrogen source, moisture content and granulometry. The best nitrogen source found was urea (5*% w/w)* along with initial moisture content of 70% and 0.85mm to 1.75mm range of

granulometry. The fish (Nile tilapia) showed 44% increase in body mass when fed with this feed. This demonstrated the capability of apple pomace to be used as fish supplement also.

2.2.5 Pigment Production

Pigments are produced by various microorganism and they find a various applications in food and textile industries. A good pigment producing microorganism should be able to use wide amount of carbon and nitrogen sources, tolerance to pH, temperature, mineral composition change and at last should provide satisfactory pigment production (Joshi et al., 2003). Apple pomace has also been used as the substrate for the production of microbial pigments in submerged fermentation. Attri et al., (2005) studied the effect of carbon and nitrogen source on carotenoids production by *Micrococcus* sp. The optimized process parameters were temperature 35°C, incubation period 96hrs and pH 6. Apple pomace (20g/l), Sodium nitrate (0.2%), fructose (0.2%) gave the maximum yield of carotenoids. Joshi et al., (2011) reported the production of carotenoids by using *Sarcina* sp. on apple pomace based medium. The use of glucose (0.1%), potassium nitrate (0.3%) gave the maximum yield. The optimum conditions giving highest concentration of carotenoids and biomass were temperature 35° C, pH 5.5, incubation period 72hrs.

2.2.6 Other products

Various other products have also been produce by SSF and SmF of apple pomace which includes heteropolysaccharide. Jin et al., (2003) produced polysaccharide-7 (PS-7) using three agro-industrial byproducts (apple juice industry byproduct, soy sauce production byproduct and Sikhye (Korean traditional food) byproduct) employing *Beijerinckia indica* in SmF. Apple pomace was found to be superior to glucose as carbon source showing highest production 4.09g/l of PS-7.

Edible mushrooms were also produced on apple pomace under solid state fermentation. Worren et al., (1992) investigated the potential of apple pomace and saw dust for the production of two edible mushrooms (Shiitake and oyster). They tested the substrate alone as well as in equal mixture. Apple pomace showed higher biomass production of mushrooms because of the optimal N level present in them.

2.3 α- amylase production by Solid-State fermentation

Alpha-amylase is one of the most industrial demanded enzymes and it is generally produced by SmF. In recent years SSF has become an alternative technique to produce the enzyme economically using agro-industrial residues (Baysal et al., 2003). Alpha amylase is used for various industrial processes performed at high temperature, so producing thermostable alpha-amylase has gained interest. Reports of producing thermostable alpha-amylase by solid state fermentation are present (Kumar et al., 2013, Ramesh et al., 1989, Sodhi et al., 2005). Various microorganisms producing alpha-amylase under SSF are-

2.3.1 Bacteria used in SSF for α-amylase production

The main group of bacteria used for alpha-amylase production is *Bacillus* species. They are known for producing thermostable alpha-amylases also (Baysal et al., 2003, Babu et al., 1995, Sodhi et al., 2005, Shukla et al., 2006).

Bacillus species grows well under solid state fermentation and produce good titer of alpha-amylase, one of such species is *Bacillus amyloliquefaciens* which is reported to produce 62470 U/g of enzyme titer at 37° C after 72 hrs of fermentation. The substrate used was the mixture of wheat bran and groundnut oil cake (1:1) which was inoculated with $2x10^9$ CFU/ml in 5gm substrate with initial moisture content of 85% (Gangadharan et al., 2006).

Other species such as *Bacillus cereus* MTCC 1305 (Anto et al., 2006), *Bacillus coagulans* have also been reported to produce alpha-amylase under SSF (Babu et al., 1995). *Bacillus subtilis, Bacillus licheniform* produces thermostable alpha amylase. They have been grown under SSF using potato peel as substrate where enzyme activity from *Bacillus subtilis* was found to be more than that of *Bacillus licheniform*. Thermal stability of the enzyme produced by *Bacillus licheniform* was found to be more with its activity been reported at 90° C (Shukla et al., 2006).

2.3.2 Fungi used in SSF for α-amylase production

Various fungi are also known to produce alpha-amylase under SSF but out of all *Aspergillus* species are the dominant ones. Fungi are grown on various agro-industrial

residues like Wheat bran, rice bran, oilseed cakes, cassava bagasse etc. *Aspergillus oryzae* var *brunneus* is widely known to produce alpha-amylase Sivaramakrishnan, et al., (2007) produced alpha-amylase on 14 agro-industrial wastes and maximum titer of enzyme (15095 U/gds) was obtained on wheat bran. Various parameters were optimized like moisture content, fermentation period, fermentation temperature, pH. Effect of various nitrogen sources (inorganic and organic both), inducers and mixture of significant substrates were also observed. Optimum conditions obtained were 72hrs fermentation period, initial moisture content 60%, 30° C fermentation temperature, pH 5. The enzyme titer increased after the addition of nitrogen source (Sodium nitrate) and C source (Starch), overall 65% of enzyme yield increased after optimization of conditions. Ramachandran et al., (2004) produced alpha amylase from coconut oil cake by *Aspergillus oryzae*. Cultivation over raw coconut oil cake gave 1372 U/gds enzyme activity. The process parameters were optimized to 30° C fermentation temperature, 72 hrs fermentation period, and 68% initial moisture content which caused the enzyme titer to reach 1827 U/gds. Addition of glucose and starch (0.5%) further increased the enzyme titer to 1911U/gds. Addition of peptone (1%) lead to 1.7 fold increase in enzyme titer (3388 U/gds)

CHAPTER 3

Materials and methods

3.1 Microorganism Used

Aspergillus oryzae was used in the present investigation to standardize various parameters for its growth and for maximization of alpha-amylase production by solid state fermentation on apple pomace.

3.2 Culture revival and inoculum preparation

The spores of *Aspergillus oryzae* were revived from freeze dried medium. Potato dextrose agar (PDA) was used for reviving and culturing of spores as shown in figure 3.5. Further sub-culturing was done to obtain pure culture.

Agar slants of PDA were prepared and inoculated with fresh fungal spores. After 7 days, 10ml of 0.1% Tween 80 was added and spores were dislodged using inoculating needle. The spore suspension was collected in sterile centrifuge tube. Spore density was set at $1x10^7$spores/ml and viability test of spores was done by inoculating them on fresh media.

3.3 Staining of fungal culture

The fungal culture was stained using Lacto-phenol cotton blue stain. Small amount of fungal culture was isolated from the test tubes having fungal slant using a stiff inoculating loop. The culture was placed on clean slide already having a drop of 70% ethanol. The material was teased gently using a needle and the slide was mounted with a drop of Lacto-phenol cotton blue reagent (Leck et al., 1999). Cover slip was placed over the material and was visualized under microscope at 40X as shown in figure 3.1.

3.4 Reagents Used

3.4.1 Salt Solution

Prepared by adding KH_2PO_4 (2g/l), NaCl (1g/l), $MgSO_4$ (1g/l) in distill water and then setting pH 5.

3.4.2 Twen-80 Solution

Prepared by adding 1ml Tween-80 in 50 ml distilled water and then making up the volume to 1000ml.

3.4.3 1% Soluble starch

Prepared by adding 1mg of starch in 10 ml distill water, heating it gently to completely dissolve the starch (clear solution) and then making up the volume to 100ml.

3.4.4 0.1% Acetate buffer

Prepared by adding 0.68 gm of sodium acetate, 0.15ml acetic acid in and then making up the volume to 50ml after setting pH 5.

3.4.5 DNS (Di-nitro Salicylic acid)

Prepared by adding 1 gm DNS to 20ml 2N NaOH and then slowly adding 30gm of Sodium potassium tartarate, and finally adding distill water to dilute the solution to 100ml.

3.4.6 Perchloric acid (52%)

3.4.7 Anthrone

Prepared by adding 20 mg of anthrone reagent in 100ml of ice-cold Sulphuric acid (95%)

3.5 Quantification of starch content in apple pomace

Apple pomace was obtained after extraction of juice manually (figure 3.1 and 3.2). It was then grinded and seived to reduce the size of particles. Grinded apple pomace (0.5 gm) as shown in figure 3.3 was mixed in 5ml 80% hot ethanol and the solution was centrifuged at 7000rpm for 12 min. Anthrone was added before draining off supernatant to check for reducing sugar and this was repeated till anthrone did not give any color. The residue after centrifugation(s) was dried in hot water bath. To residue 5ml water and 6.5ml 52% perchloric acid was added and the solution was kept at 0°C for 20min. It was then centrifuged at 7000rpm for 12min and supernatant was transferred to new tube, the process was repeated thrice and then water was added to make the volume of supernatant 100ml. 0.1ml of supernatant solution was taken and volume was made up to 1ml, standard of glucose were prepared and to each tube 4 ml of anthrone was added and heated in boiling water bath for 8 to 10 min.

The anthrone solutions were rapidly cool and the readings were taken at 630nm (Yemm., 1954). To calculate the starch content concentration of reducing sugar was multiplied by the 0.9.

3.6 Quantification of reducing sugars in fermented apple pomace

After the fermentation DNS test of each sample was performed to estimate the amount of reducing sugar present in the fermented apple pomace. Fermented solution was prepared by as described in topic 3.9, this solution diluted 100 times and standard of glucose were prepared (0, 0.2, 0.4, 0.6, 0.8, 1.0) mg/ml. To each tube 3ml of DNS was added and heated at boiling water bath for 10 min then O.D of the solutions were taken at 540nm. The concentration of reducing sugar was calculated using the standard curve.

3.7 Enzyme assay of α-amylase

α-amylase enzyme assay was done according to Bernfeld et al., (1955). To each test tube 1.25 ml of soluble starch, 0.5ml of 0.1M acetate buffer (pH5) and 10 times diluted crude extract (fermented solution) were added. The solution was incubated at 50° C for 10 min, after the incubation DNS of the solution was performed. Activity of the enzyme was calculated by-

$$Enzyme\ activity = \frac{\mu Mol\ of\ product\ formed \times Total\ volume}{Incubation\ time(\text{min}) \times \text{ml of crude enzyme extract}}$$

3.8 Statistical analyses

All the experiments were done in triplicate replication and data were analyzed using SPSS 16 software. One way analysis of variance (ANOVA) was used to assess the differences among the treatments of various experiments and the treatment were compared at <0.05 P level of significance. In case, the ANOVA effects were important, assessments between the different means were made to quantify and evaluate the source of variation, and critical differences and standard error of means values were calculated at <0.05 P level.

3.9 Method used to set up SSF in various experiments

In an Erlenmeyer flask 10gm of Apple pomace was taken and to it 2ml of salt solution was added along with distill water to make the moisture content 60% (if not specified).

The flask was then autoclaved at 121° C for 15 min and inoculated with 2ml of spore suspension ($1x10^7$spores/ml). Various parameters were optimized like fermentation time, moisture content, best nitrogen source and mixture of other agro-industrial wastes employing the same reaction.

Fermented solution was prepared by adding 100 ml of 0.1% Tween-80 to each flask after the completion of fermentation and by shaking them at 160rpm for 60min. The solution was transferred to centrifuge tubes and centrifuged at 15000rpm for 15 min, Supernatant was kept and the residue was discarded. Supernatant was divided into two parts one of which was subjected to reducing sugar estimation as described in 3.6 and enzyme assay was performed of the other part as described in 3.7.

The reducing sugar obtained by method 3.6 was subtracted from the sugar obtained by method 3.7 and that will determine the actual amount of sugar produced by enzyme.

Figure 3.1

Figure 3.1 & 3.2 Apple pomace before sieving and crushing

Figure 3.3- Apple pomace after crushing and seiving

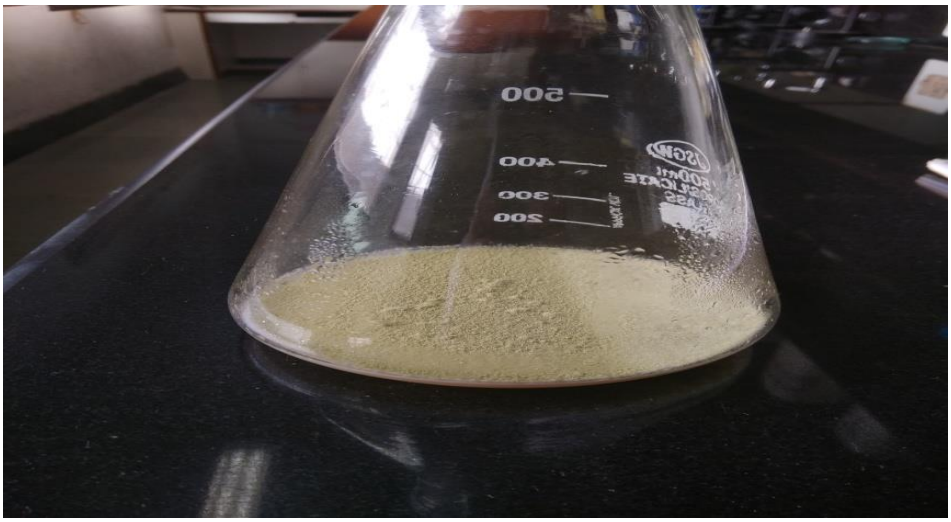

Figure 3.4- Spore viability test to check the viability and re-growth of spores on PDA media

3.10 Experiment 1- Optimization of initial moisture content.

This experiment was performed to evaluate the best moisture content of the substrate for the growth and production of enzyme by the fungus. In different flasks 10gm of apple pomace was taken, 2 ml of salt solution was added and appropriate amount of water to make the initial moisture content as 40%, 50%, 60%, 70%, 80% (Treatments in this

experiment). To every flask 2ml of spore solution ($1x10^7$spores/ml) was inoculated and incubated for 96 hrs at 30° C. After the fermentation, enzyme assay of the solution was done.

3.11 Experiment 2- Optimization of fermentation time.

This experiment was performed to optimize the time taken by fungus to produce highest titer of enzyme. In different flasks 10 gm apple pomace was taken, 2ml of salt solution was added and appropriate amount of water to make the moisture content 70% (as resulted in previous experiment). To the flasks 2ml of spores ($1x10^7$spores/ml) were inoculated and incubated for 24hrs, 48hrs, 72hrs, 96hrs, 120hrs and 144hrs (Different treatment in this experiment). After the fermentation enzyme assay of the solution was done.

3.12 Experiment 3- Optimization of pH of the substrate

The objective of this experiment was to find out the most suitable pH of the substrate for the growth and production of enzyme by the fungus. In different flask 10gm of apple pomace was taken, 2ml of salt solution was added and apt quantity of water to make the initial moisture content 70%. The pH of the salt solution and water was varied as 3, 4, 5, 6, 7, 8 (treatment in this experiment) and 2ml of spores ($1x10^7$spores/ml) were inoculated. Fermentation was carried out for 96 hrs and enzyme assay was performed at the end.

3.13 Experiment 4- Optimization of nitrogen source

The experiment was performed to evaluate the effect of different inorganic nitrogen sources on enzyme production. The experiment was performed using the optimum conditions as resulted from above experiments (70% moisture content, 96hr fermentation time, pH 5). Various nitrogen sources Ammonium Phosphate, Ammonium Sulphate, Ammonium Nitrate, Ammonium Chloride and Sodium Nitrate were added at a concentration of 0.25M. Enzyme assay was performed after the 96 hrs of fermentation.

3.14 Experiment 5- Optimization of different substrate combination with apple pomace (1:1).

The experiment was conducted to find out the other agro-industrial wastes which could be used in combination with apple pomace to increase the productivity of the enzyme**.** Various substrates that were used with apple pomace in 1:1 ratio were Mustard oil cake, Rice husk, Potato peels and Wheat bran. To different flasks 5gm of apple pomace was added along with 5gm of respective substrate. The conditions were optimized as found out in above experiments and enzyme assay was performed at the end of fermentation. The significant substrate combinations were further evaluated to optimize the best ratio for enzyme production.

3.15 Experiment 6- Optimization of different ratio of potato peel with apple pomace

The experiment was performed to find out the best ratio of potato peel with apple pomace to get highest yield of enzyme. To different flask various combination of potato peel and apple pomace were added in this ratio 1:1, 1:2, and 1:3 to make total weight of substrate 10gm. All the fermentation conditions were kept same as optimized in previous experiments. Enzyme assay was performed after the completion of fermentation.

3.16 Experiment 7- Optimization of different ratio of wheat bran with apple pomace

The experiment was performed to find out the best combination of wheat bran with apple pomace to get the best yield of enzyme. To different flasks various combinations of wheat bran and apple pomace were added as 1:1, 1:2, and 1:3 to make the total weight of substrate 10gm. All the fermentation conditions were kept same as optimized in above experiments. Enzyme assay was performed after the completion of fermentation.

3.17 Observation recorded

3.17.1 Fungal culture

Aspergillus species have a characteristic feature of aspergillum like spore bearing structure. Distinct single conidiophores are also correspondent to *Aspergillus* species. The rounded club shaped spore bearing structure is also a feature associated with this species. All these features are seen in figure 3.5.

The stained fungus observed under microscope (40X) after staining.

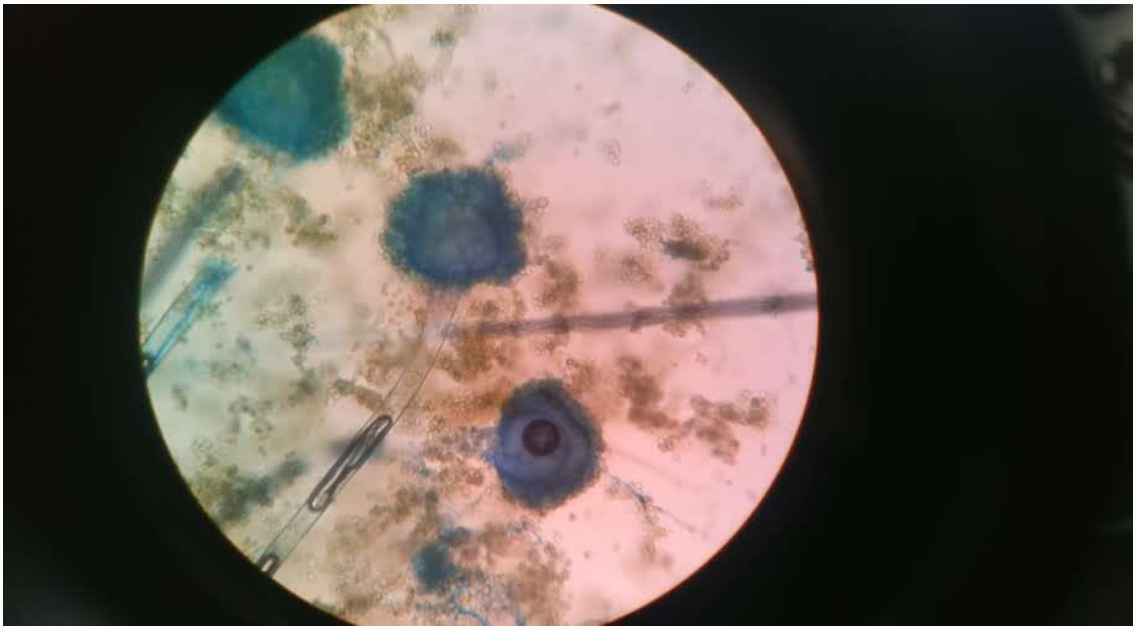

Figure 3.5- Fungus observed under 40X magnification after LCB staining.

3.17.2 Spore Count

Spore count observation was performed using haemocytometer. The spores collected in centrifuge tube after dislodging from the culture slants were observed. The spore density to be set was $1x10^7$spores/ml, so dilution was done to set the required density. Direct microscopic spore count by Neubauer haemocytometer was performed using microscope.

$$Conc.of\ Spores = \frac{No.of\ cells\ counted}{Chamber\ counted \times Volume\ of\ chamber}$$

CHAPTER 4

Results and Discussion

4.1 Starch content in apple pomace

The amount of starch found in the apple pomace was around 11.88 ± 0.37% which is comparable to normal level of starch in apple pomace (Gullón et al., 2007). Apple pomace lots used in this study in course of time did not show any variation in the amount of starch content. Table 4.1 depicts the amount of starch content in different lots of apple pomace used. Starch content is one of the major factors which could influence the production of α-amylase

Table 4.1: Starch content analyzed in apple pomace which were used in SSF

Sample	Total starch in 100ml of solution(µg)	Starch (mg)	Percentage of starch (%)
Lot 1	61571.39	61.571	12.31
Lot 2	57373.09	57.373	11.47
Lot 3	58162.77	58.16277	11.63255

4.2 Optimization of moisture content

The differences in enzyme activity recorded under various treatment used to optimize initial moisture content of apple pomace substrate were significant at <0.05P level of significance (Table 4.2). The initial moisture content of apple pomace adjusted at 70% showed the maximum enzyme activity after three days of fermentation. The enzyme activity was almost similar on 40%, 50% and 60% moisture content. It reached a maximum of 4449.233± 34.97 U/gds at 70% moisture content and then declined at 80%

moisture content which suggests that 70% is the optimum moisture content required for the growth of fungus (Figure 4.1 and 4.2). *Aspergillus oryzae* is known to grow at above 60% of moisture content in SSF (Francis et al, 2002).

Table 4.2: Mean enzyme activity of α-amylase at different moisture content in apple pomace after SSF by *Aspergillus oryzae.*

S.No.	Moisture content in A.P	Enzyme Activity (U/gds)*
1	40%	3828.779± 284.88 [a]
2	50%	3757.772± 46.94 [a]
3	60%	3592.845± 93.51 [a]
4	70%	4449.233± 34.97 [b]
5	80%	3896.2±324.47 [a]

*Significant level at p<0.05.

a-b = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

4.3 Optimization of fermentation time

The optimum fermentation time for maximum enzyme production was found out to be 96hrs with 70% moisture content. The enzyme activity found at 96 hrs was 6731.47±26.21 U/gds which was better than at 48, 72, 120 and 144hrs (Figure 4.3 and 4.4). The enzyme activity gradually increased day by day from 48hrs (883.79±55U/gds) reaching a maximum on 96hrs and the decreasing further afterwards as seen in Table 4.3. Table 4.3 also shows that the various treatments used to optimize fermentation time had differences in enzyme activities which were significant at <0.05P level of significance (Table 4.3). *Aspergillus oryzae* generally takes three to four day to grow on the substrate in SSF (Battaglino et al., 1991).

Table 4.3: Mean enzyme activity of α-amylase at different fermentation time obtained under SSF of apple pomace by *Aspergillus oryzae.*

S.No.	Fermentation time	Enzyme activity (U/gds)*
1	48hrs	883.79±55 [a]
2	72hrs	5105.11± 31.38 [b]
3	96hrs	6731.47±26.21 [c]
4	120hrs	3076.53±26.56 [d]
5	144hrs	530.25±26.52 [e]

*Significant level at p<0.05.

a-e = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

4.4 Optimization of pH

The differences in the enzyme activity of α-amylase obtained at different pH of apple pomace were significant at <0.05P level of significance (Table 4.4). The optimum pH for the maximum enzyme production was found out to be pH 5 with 70% moisture content and 96hrs of fermentation. Figure 4.5 and 4.6 shows that although the enzyme activity at pH 6 is similar to that of pH 5 but it was observed that the growth of fungus was better at pH 5. The data pertaining to enzyme activity observed at different pH is presented in table 4.4 where the maximum enzyme activity was at pH 5 (5007.93±185.48 U/gds). There was very less growth observed at pH 3, 4 and 8 which could be because of extreme condition for the fungus to grow. pH 5 and 6 showed extensive growth , with slightly moderate growth at pH 7.

Table 4.4: Mean enzyme activity of α-amylase at different pH of apple pomace under SSF by *Aspergillu oryzae*.

S.No.	pH	Enzyme activity (U/gds) *
1	pH3	3901.85±52.09 [a]
2	pH4	3982.17±269.34 [a]
3	pH5	5007.93±185.48 [b]
4	pH6	4974.44±249.88 [b]
5	pH7	3884.98±41.13 [c]
6	pH8	2949.87±140.56 [d]

*Significant level at p<0.05.

a-d = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

4.5 Optimization of Nitrogen Source

Out of different inorganic nitrogen sources supplemented with apple pomace, the enzyme activity of α-amylase was found to be highest in Sodium Nitrate (0.25 M). The fungus showed extensive growth in the substrate having Sodium nitrate as nitrogen source when compared to other nitrogen sources. Different enzyme activities were obtained under various nitrogen sources as seen in table 4.5. Most of the nitrogen sources used were ammonium salts so a conclusion can be made that ammonium salts are not the best nitrogen sources for *Aspergillus oryzae.* Substrate having nitrogen sources as ammonium sulphate, ammonium nitrate, ammonium chloride and ammonium phosphate showed almost similar enzyme activity (Figure 4.7 and 4.8) . The differences observed in enzyme activity under different nitrogen sources supplementation were significant at <0.05 P level of significance (Table 4.5).

Table 4.5: Mean enzyme activity of α-amylase obtained under SSF of apple pomace supplemented with various nitrogen sources at 0.25 M.

S.No	Nitrogen Source	Enzyme activity (U/gds)*
1	Ammonium Sulphate	4271.2±161.40 [a]
2	Ammonium Nitrate	5157.4±309.82 [b]
3	Ammonium Phosphate	4356±78.25 [ac]
4	Sodium Nitrate	6475.8±149.93 [d]
5	Ammoinium Chloride	4600±89.59 [abc]

*Significant level at p<0.05.

a-d = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

4.6 Optimization of different substrate combination with apple pomace (1:1).

On perusal of data presented in table 4.6, it is evident that the enzyme activity of α-amylase obtained by using the combination of apple pomace with wheat bran at 1:1 ratio was the best when compared to other combinations. The enzyme activity of wheat bran: Apple pomace (1:1) was found to be 7206.6±371.7 U/gds. Other substrates used were potato peel, mustard oil cake and rice husks. Potato peel with apple pomace at 1:1 also showed significant enzyme activity when compared to other two sources which could be seen from figure 4.9 and 4.10. The enzyme activity of potato peel: apple pomace (1:1) was found to be 5565.5±654.084 U/gds. Ratios of potato peel and wheat bran with apple pomace were further optimized for best enzyme activity. The differences in the enzyme activity obtained by SSF of apple pomace with different substrate were significant at <0.05P level of significance (Table 4.6).

Table 4.6: Mean enzyme activity of α-amylase obtained after SSF of apple pomace supplemented with other substrates in 1:1 ratio.

S.No	Substrates Ratio	Enzyme acitvity(U/gds)*
1	Mustard oil cake: Apple pomace (1:1)	1383.3±82.34 [a]
2	Rice Husk: Apple pomace (1:1)	886.24±13.12 [a]
3	Potato peel: Apple pomace (1:1)	5565.5±654.084 [b]
4	Wheat Bran: Apple pomace (1:1)	7206.6±371.7 [c]

*Significant level at p<0.05.

a-c = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

4.7 Optimization of different ratio of potato peel with apple pomace

From the table 4.7 it is apparent that the ratio (1:2) of potato peel with apple pomace showed the best enzyme activity when compared to other combinations of 1:1 and 1:3. The enzyme activity was found to be 8582±1.34 U/gds. Higher enzyme activity at 1:2 ratio could be linked with better growth of fungus which was observed at this combination as compared to other combinations. It can be concluded that apple pomace is acting as the source of freely available sugar which supports the growth of fungus and potato peels acts as rich source of starch which is facilitating the production of enzyme. Different enzyme activity of α-amylase that were obtained at different ratio of potato peel with apple pomace are significant at <0.05 P level of significance (Table 4.7). Figure 4.12 depicts the graphical representation of mean enzyme activity at different ratios. Figure 4.11 shows the variation in the enzyme activity of triplicates used for different treatments.

Table 4.7- : Mean enzyme activity of α-amylase at various ratios of Potato peel with apple pomace obtained under SSF of apple pomace by *Aspergillus oyzae.*

S.No	Ratios	Enzyme acitivity (U/gds)*
1	PP:AP(1:1)	5565.5±82.34 [a]
2	PP:AP(1:2)	8582±1.34 [b]
3	PP:AP(1:3)	6896.9±67.13 [ab]

*Significant level at p<0.05.

a-b = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

4.8 Various combinations of wheat bran with apple pomace

The maximum enzyme activity of α-amylase obtained by combining wheat bran with apple pomace was at 1:1 ratio which could be inferred from figure 4.14. The enzyme activity at 1:1 ratio was 7206.6±371.7 U/gds. Other ratio of wheat bran with apple pomace showed good growth but not very high enzyme activity. We can conclude that apple pomace being a rich source of reducible sugar supported the growth of fungus and wheat bran being the rich source of starch lead to high enzyme production. The enzyme activity recorded under various ratios were significantly different at <0.05P level of significance (Table 4.8).

Table 4.8: Mean enzyme activity of α-amylase showing different ratio of wheat bran with apple pomace after SSF by *Aspergillus oryzae*

S.No.	Ratio	Enzyme activity (U/gds)*
1	WB:AP (1:1)	7206.6±371.7 [a]
2	WB:AP (1:2)	5915±218.85 [b]
3	WB:AP (1:3)	4657.4±60.86 [c]

*Significant level at p<0.05.

a-c = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

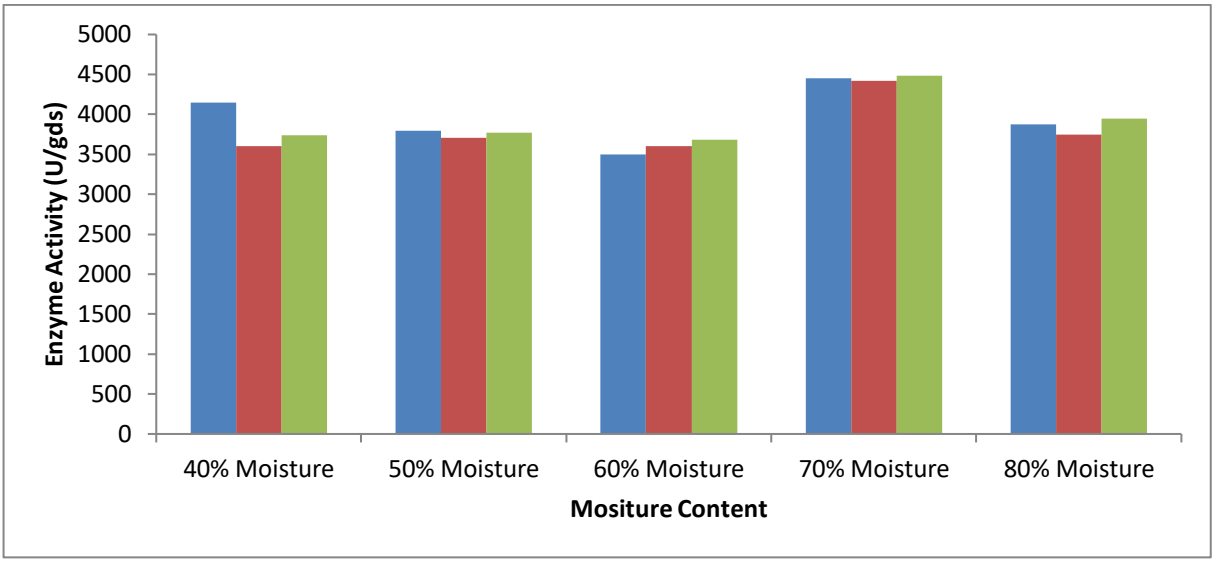

Figure 4.1: Enzyme activity of α-amylase in triplicates obtained at different moisture ratio of Apple pomace by *Aspergillus oryzae.*

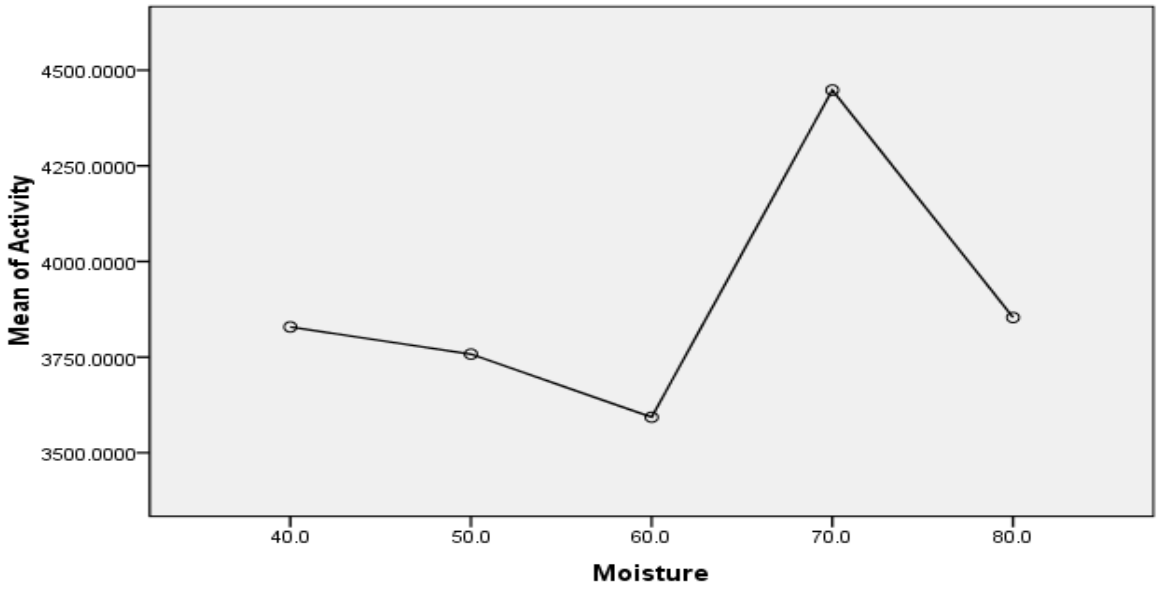

Figure 4.2: Mean enzyme activity of α-amylase depicting the best moisture ratio (70%) for enzyme production.

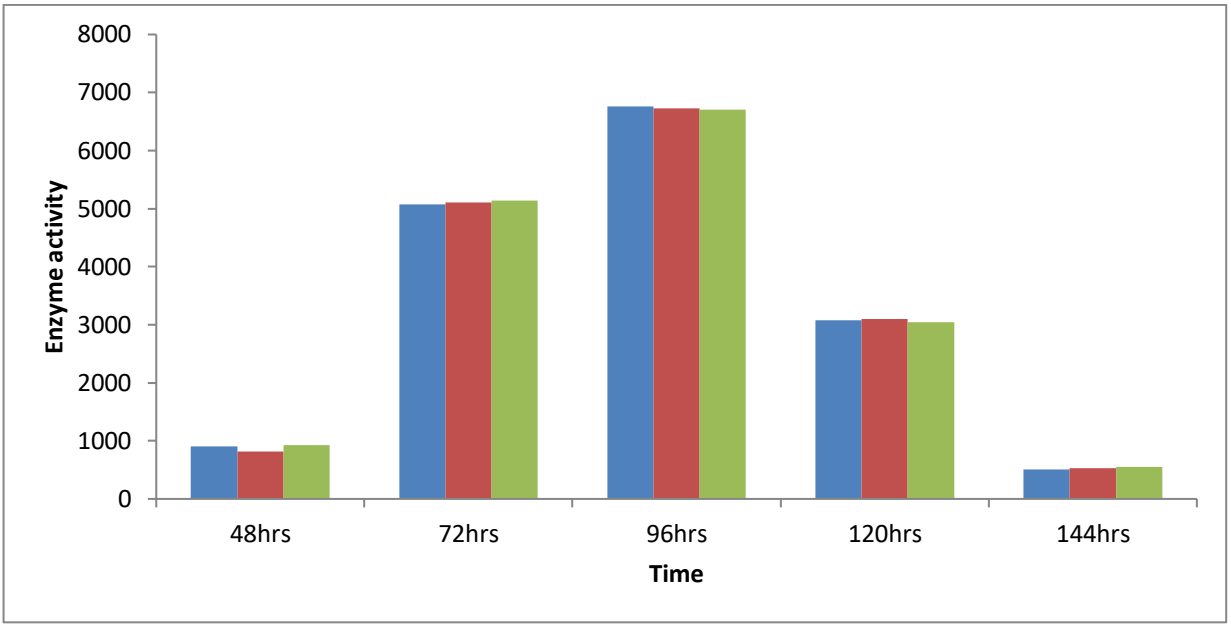

Figure 4.3: Enzyme activity of α-amylase in trplicates obtained after different fermentation time of Apple pomace under SSF by *Aspergillus oryzae*.

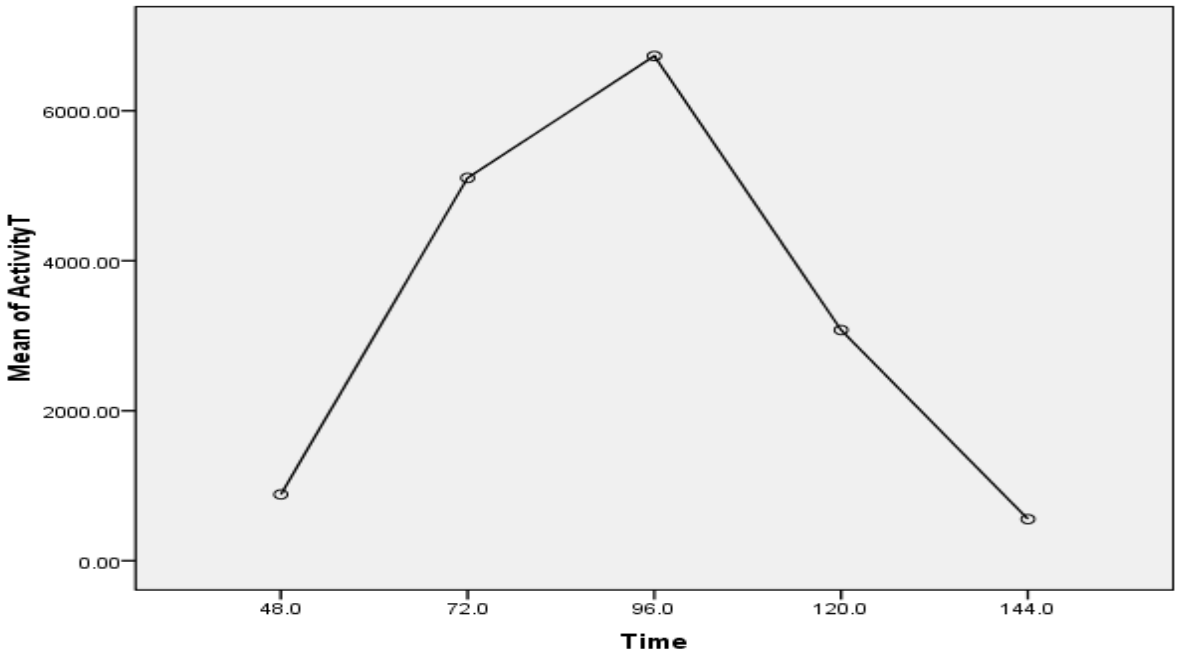

Figure 4.4: Mean enzyme activity of α-amylase showing the best fermentation time (96hrs) of apple pomace by *Aspergillus oryzae*

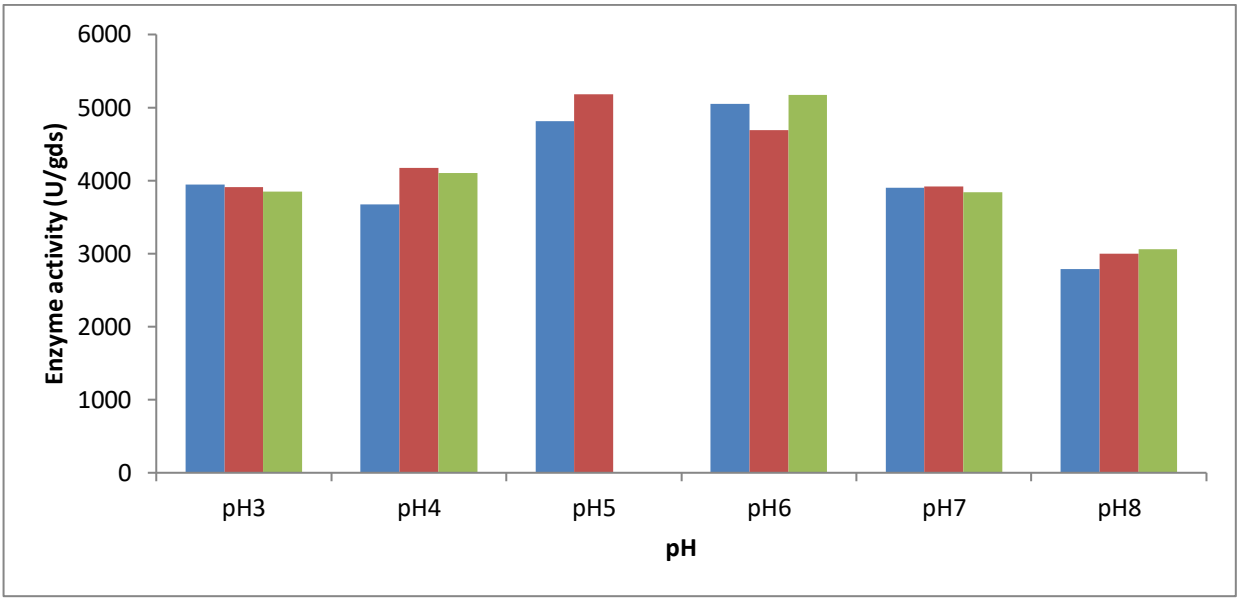

Figure 4.5: Enzyme activity of α-amylase in triplicates obtained after different pH after fermentation of Apple pomace by *Aspergillus oryzae.*

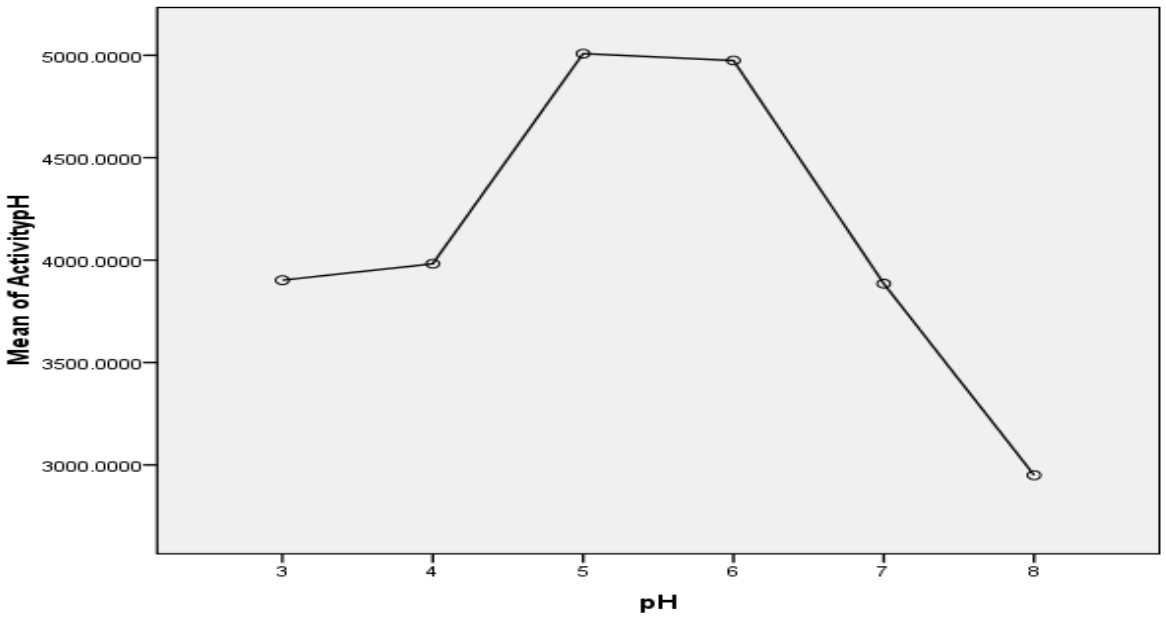

Figure 4.6: Mean enzyme activity of α-amylase showing the best pH (pH5) for the fermentation of apple pomace by *Aspergillus oryzae.*

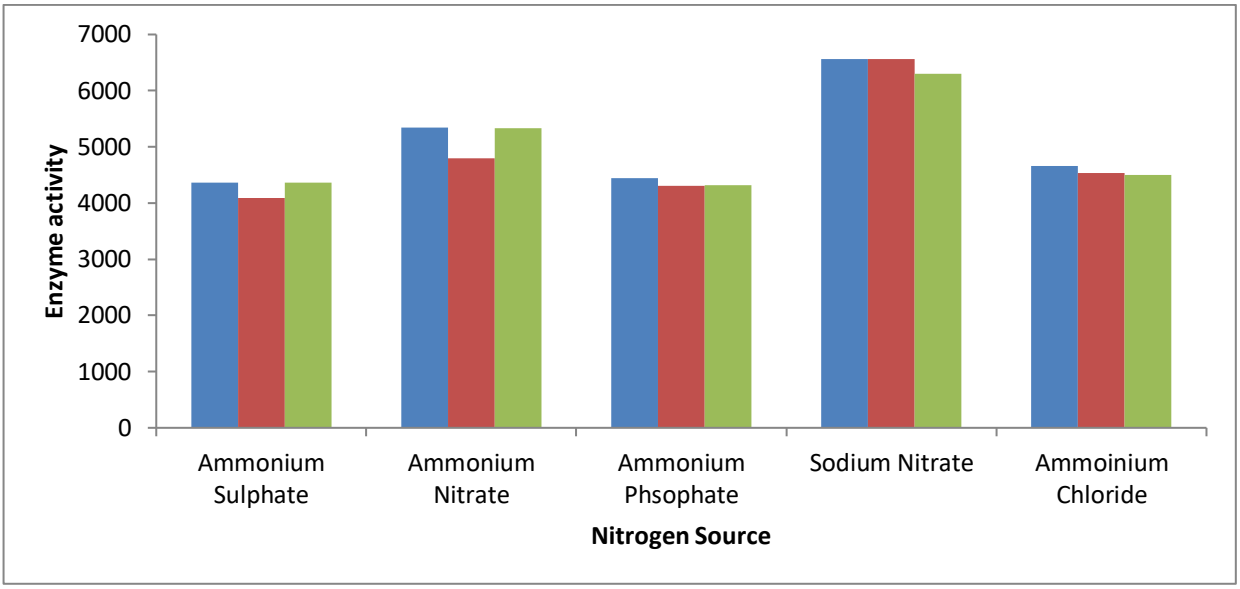

Figure 4.7: Enzyme activity of α-amylase in triplicates obtained after supplementation of different nitrogen sources (0.25M) with apple pomace and fermentation by *Aspergillus oryzae.*

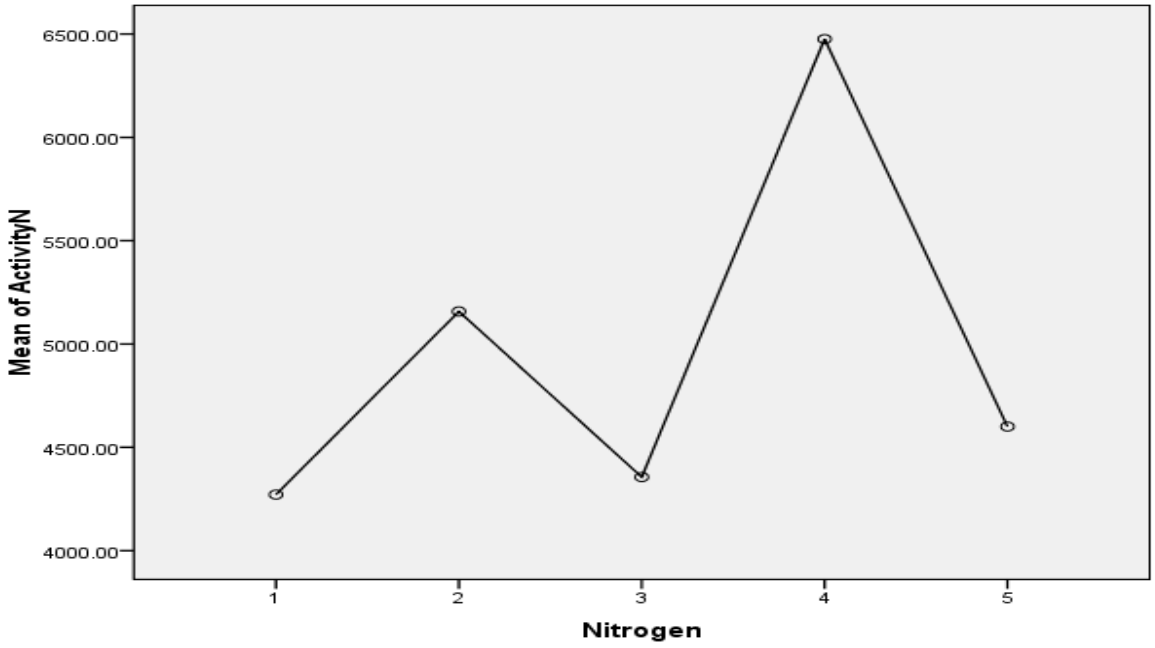

Figure 4.8: Mean enzyme activity of α-amylase showing the best pH for the fermentation of apple pomace by *Aspergillus oryzae* (1- Ammonium sulphate, 2- Ammonium Nitrate, 3- Ammonium phosphate, 4- Sodium Nitrate, 5- Ammonium chloride).

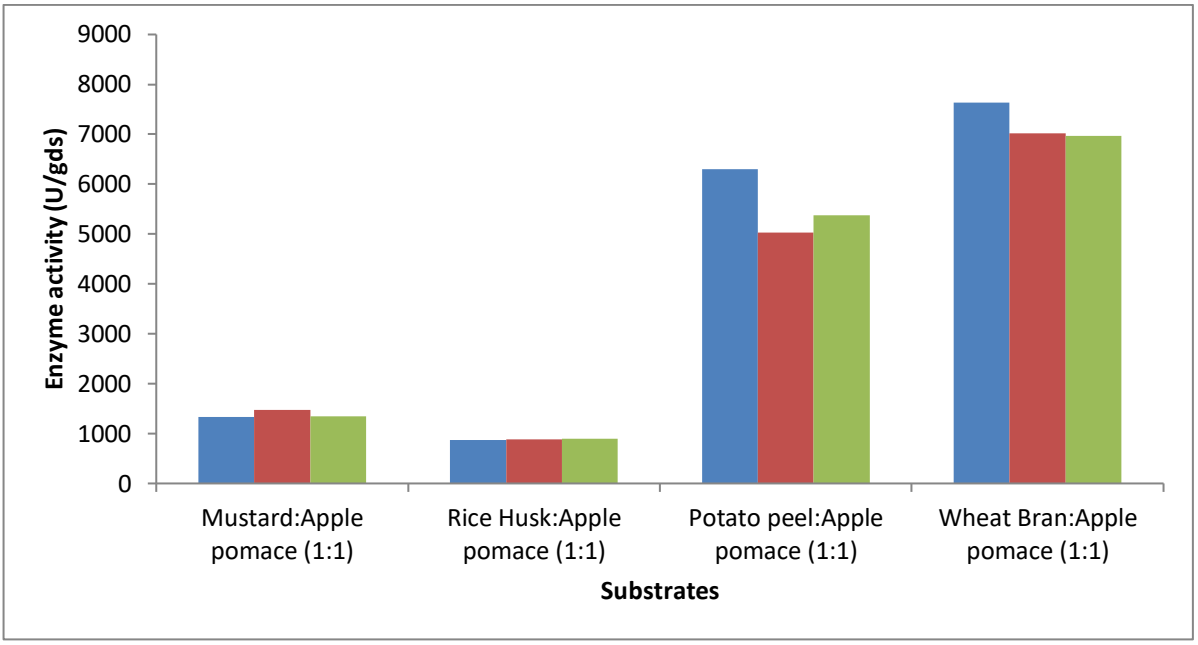

Figure 4.9: Enzyme activity of α-amylase in triplicates when different substrates are mixed with apple pomace at 1:1 ratio.

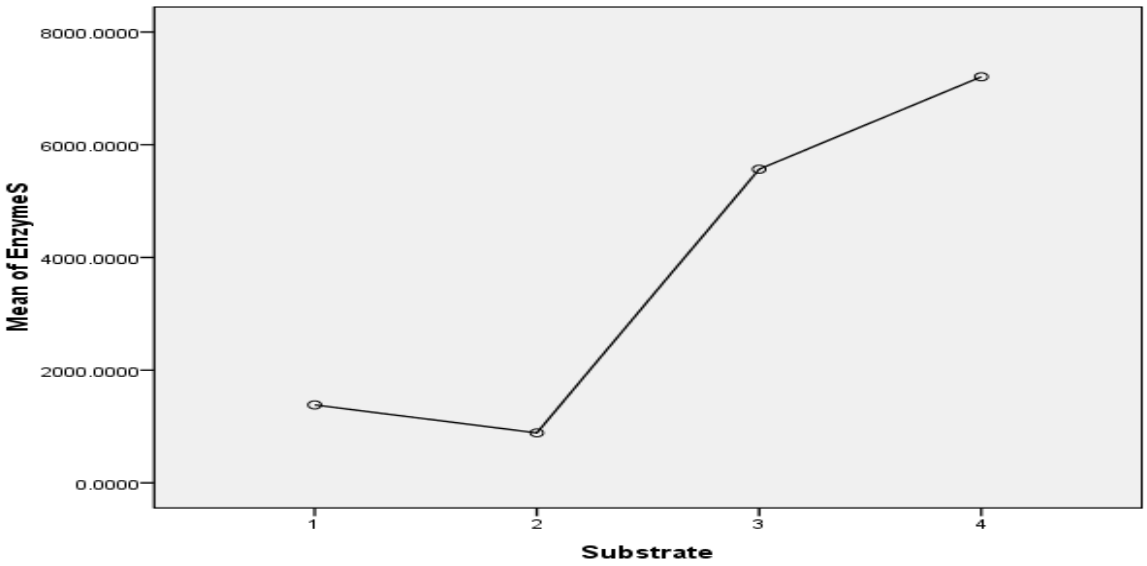

Figure 4.10: Mean enzyme activity of α-amylase showing different substrate mixed with apple pomace in 1:1 ratio after fermentation by *Aspergillus oryzae* (1- Mustard oil cake: Apple pomace (1:1), 2- Rice husks: Apple pomace (1:1), 3- Potato peels: Apple pomace (1:1), 4- Wheat bran: Apple pomace (1:1).

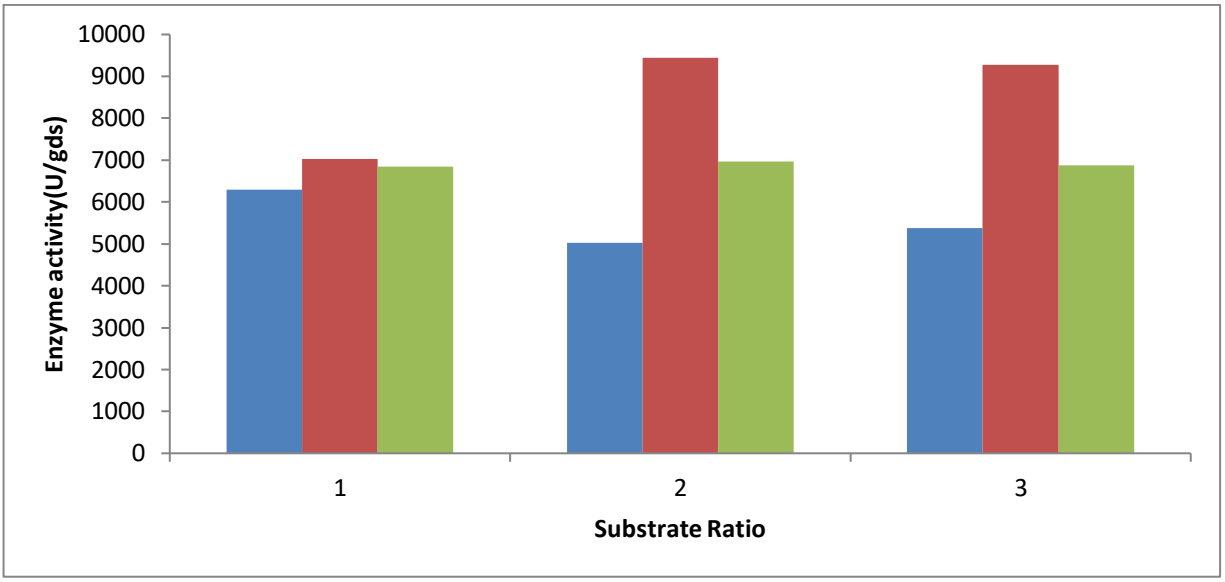

Figure 4.11: Enzyme activity of α-amylase in triplicates obtained after different ratios of potato peels with apple pomace (1- potato peel: apple pomace (1:1), 2- Potato peel: apple pomace (1:2), 3- Potato peel: apple pomace (1:3)) after fermentation of Apple pomace by *Aspergillus oryzae.*

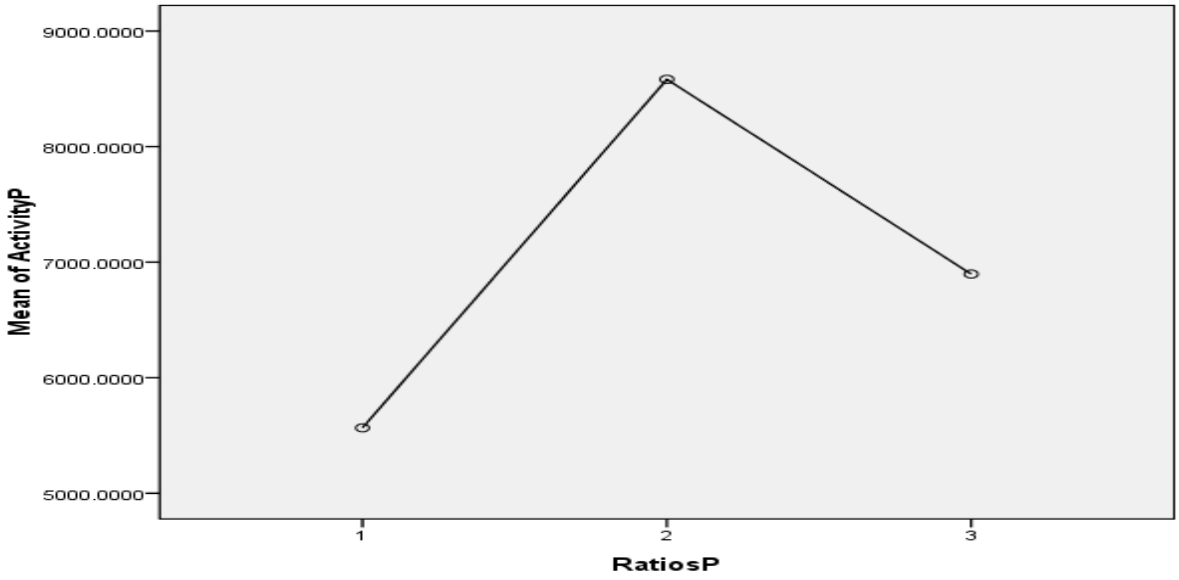

Figure 4.12: Mean enzyme activity of α-amylase showing different ratio of potato peels with apple pomace after fermentation by *Aspergillus oryzae* (1- Potato peel: apple pomace (1:1), 2- Potato peel: apple pomace (1:2), 3- Potato peel: apple pomace (1:3)).

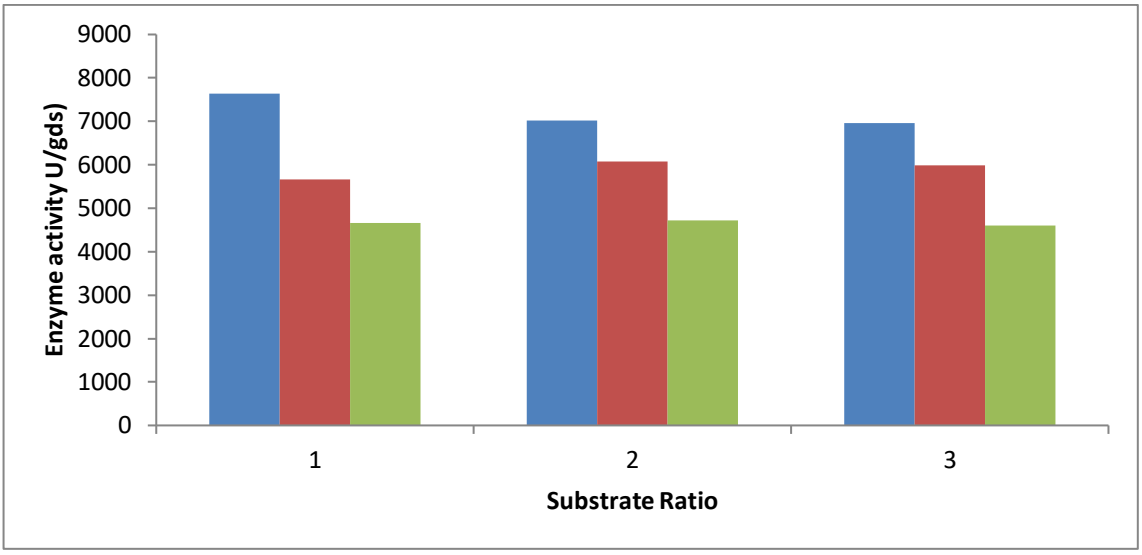

Figure 4.13: Enzyme activity of α-amylase in triplicates obtained after different ratios of wheat bran with apple pomace (1- Wheat bran: apple pomace (1:1), 2- Wheat bran: apple pomace (1:2), 3- Wheat bran: apple pomace (1:3)) after fermentation of Apple pomace by *Aspergillus oryzae.*

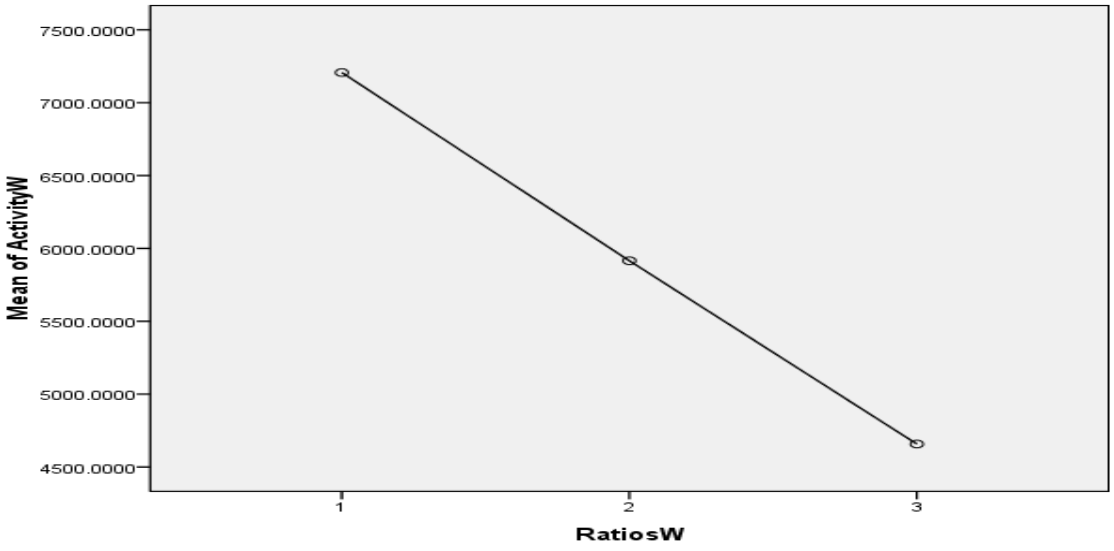

Figure 4.14: Mean enzyme activity of α-amylase showing different ratio of wheat bran with apple pomace after fermentation by *Aspergillus oryzae* (1- Wheat bran: apple pomace (1:1), 2- Wheat bran: apple pomace (1:2), 3- Wheat bran: apple pomace (1:3)).

Table 4.9: Effect of different parameters (Fermentation time, Moisture content, pH and various substrate ratios) on enzyme activity of α-amylase produced by *Aspergillus oryzae* under SSF of apple pomace.

Fermentation Time	**Enzyme activity (U/gds) ***	**Moisture content**	**Enzyme activity (U/gds) ***	**pH**	**Enzyme activity (U/gds) ***	**Substrate ratios (1:1) with A.P**	**Enzyme acitivity (U/gds) ***
48hrs	883.79±55 [a]	40%	3828.779±[f] 284.88	3	3901.85± [h] 52.09	Mustard oil cake	1383.3±[k] 82.34
72hrs	5105.11±[b] 31.38	50%	3757.772± [f] 46.94	4	3982.17± [h] 269.34	Rice Husk	886.24± [k] 112
96hrs	6731.47± [c] 26.2 1	60%	3592.845± [f] 93.51	5	5007.93± [i] 185.48	Potato peel	5565.5± [l] 654.084
120hrs	3076.53± [d] 26.56	70%	4449.233±[g] 34.97	6	4974.44± [i] 249.88	Wheat Bran	7206.6± [m] 371.7
144hr	530.25± [e] 26.52	80%	3896.2± [f] 324.47	7	3884.98± [h] 41.13		
				8	2949.87± [j] 140.56		

*Significant level at $p<0.05$.

a-m = Means in the column with same superscript letters are not significantly different as measured by Tukey's-HSD range test between isolate

Chapter 5
Conclusion

This study was conducted to optimize various conditions and parameters for α-amylase production via solid state fermentation of apple pomace and other substrates when supplemented with apple pomace. The best combination of parameters were moisture ratio 70%, fermentation time 96 hrs, pH 3, sodium nitrate as nitrogen source (0.25 M), when inoculated of 2ml ($1x10^7$spores/ml) at 30° C as shown in table 4.9.

The enzyme activity obtained by combining these conditions was found to be 6475±149.93 U/gds. The enzyme activity obtained is comparable to other substrates and fungus used for the production of α-amylase under solid state fermentation (Singh et al., 2014).

Among four of agro-industrial waste tested, supplementation of wheat bran and potato peels with apple pomace were the most suitable both in terms of enzyme production and their availability. Both these sources are rich in starch content which also rationalize their use with apple pomace. The best ratios of these substrates with apple pomace were evaluated. Potato peel with apple pomace at a ratio of 1:2 and Wheat bran with apple pomace at a ratio of 1:1 were found optimum in terms of fungal growth and enzyme production.

The completely optimized SSF process for α-amylase production if validated at small scale fermentor and industrial level will provide an opportunity for value addition of apple pomace. This could ultimately result in additional income source of farmers and local people.

References

A. Almosnino, M. Bensoussan and J. Belin, "Unsaturated fatty acid bioconversion by apple pomace enzyme system. Factors influencing the production of aroma compounds", *Food Chemistry*, vol. 55, no. 4, pp. 327-332, 1996.

A. Almosnino, M. Bensoussan and J. Belin, "Unsaturated fatty acid bioconversion by apple pomace enzyme system. Factors influencing the production of aroma compounds", *Food Chemistry*, vol. 55, no. 4, pp. 327-332, 1996.

A. Pandey, C. Soccol and D. Mitchell, "New developments in solid state fermentation: I-bioprocesses and products", *Process Biochemistry*, vol. 35, no. 10, pp. 1153-1169, 2000.

Albuquerque, Patrícia Melchionna. "Estudo da produção de proteína microbiana a partir do bagaço de maçã." (2003).

Anto, Hema, Ujjval Trivedi, and Kamlesh Patel. "α-Amylase production by Bacillus cereus MTCC 1305 using solid-state fermentation." *Food Technology and Biotechnology* 44.2 pp: 241-245, 2006

Attri, Devender, and V. K. Joshi. "Optimization of apple pomace based medium and fermentation conditions for pigment production by Micrococcus species." 2005.

B. Gullón, E. Falqué, J. Alonso and J. Parajó, "Evaluation of Apple Pomace as a Raw Material for Alternative Applications in Food Industries", *Hrcak.srce.hr*, 2018.

B. Gullón, R. Yáñez, J. Alonso and J. Parajó, "l-Lactic acid production from apple pomace by sequential hydrolysis and fermentation", *Bioresource Technology*, vol. 99, no. 2, pp. 308-319, 2008..

Baysal, F. Uyar and Ç. Aytekin, "Solid state fermentation for production of α-amylase by a thermotolerant Bacillus subtilis from hot-spring water", *Process Biochemistry*, vol. 38, no. 12, pp. 1665-1668, 2003.

Bernfeld, Peter. "[17] Amylases, α and β." *Methods in enzymology* 1 (1955): 149-158.

Berovič and H. Ostroveršnik, "Production of Aspergillus niger pectolytic enzymes by solid state bioprocessing of apple pomace", *Journal of Biotechnology*, vol. 53, no. 1, pp. 47-53, 1997.

Bioethanol Production from Apple Pomace left after Juice Extraction", *The Internet Journal of Microbiology*, vol. 5, no. 2, 2008.

E. Yemm and A. Willis, "The estimation of carbohydrates in plant extracts by anthrone", *Biochemical Journal*, vol. 57, no. 3, pp. 508-514, 1954.

F. Francis, A. Sabu, K. Nampoothiri, G. Szakacs and A. Pandey, "Synthesis of -amylase by Aspergillus oryzae in solid-state fermentation", *Journal of Basic Microbiology*, vol. 42, no. 5, pp. 320-326, 2002.

F. Vendruscolo, C. da Silva Ribeiro, E. Esposito and J. Ninow, "Protein Enrichment of Apple Pomace and Use in Feed for Nile Tilapia", *Applied Biochemistry and Biotechnology*, vol. 152, no. 1, pp. 74-87, 2008.

G. Dhillon, S. Brar, M. Verma and R. Tyagi, "Enhanced solid-state citric acid bio-production using apple pomace waste through surface response methodology", *Journal of Applied Microbiology*, vol. 110, no. 4, pp. 1045-1055, 2011.

G. Dhillon, S. Kaur, S. Brar, F. Gassara and M. Verma, "Improved xylanase production using apple pomace waste by Aspergillus niger in koji fermentation", *Engineering in Life Sciences*, vol. 12, no. 2, pp. 198-208, 2012.

Gangadharan, Dhanya, et al. "Solid Culturing of Bacillus amyloliquefaciens for Alpha Amylase Production." *Food Technology & Biotechnology* pp.44.2 2006.

H. Jin, H. Kim, S. Kim, M. Shin, J. Kim and J. Lee, "Production of heteropolysaccharide-7 by Beijerinckia indica from agro-industrial byproducts", *Enzyme and Microbial Technology*, vol. 30, no. 6, pp. 822-827, 2002.

H. Rahmat, R. Hodge, G. Manderson and P. Yu, "Solid-substrate fermentation ofKloeckera apiculata andCandida utilis on apple pomace to produce an improved

stock-feed", *World Journal of Microbiology & Biotechnology*, vol. 11, no. 2, pp. 168-170, 1995.

H. Sodhi, K. Sharma, J. Gupta and S. Soni, "Production of a thermostable α-amylase from Bacillus sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production", *Process Biochemistry*, vol. 40, no. 2, pp. 525-534, 2005.

H. Sun, X. Ge, Z. Hao and M. Peng, "Cellulase production by Trichoderma sp . on apple pomace under solid state fermentation", *Ajol.info*, 2018.

HANG, C. LEE and E. WOODAMS, "A Solid State Fermentation System for Production of Ethanol from Apple Pomace", *Journal of Food Science*, vol. 47, no. 6, pp. 1851-1852, 1982.

Hang, Y. D., et al. "Production of alcohol from apple pomace." *Applied and environmental microbiology* 42.6 (1981): 1128-1129.

J. Shukla and R. Kar, "Potato peel as a solid state substrate for thermostable α-amylase production by thermophilic Bacillus isolates", *World Journal of Microbiology and Biotechnology*, vol. 22, no. 5, pp. 417-422, 2006.

J. Worrall and C. Yang, "Shiitake and Oyster Mushroom Production on Apple Pomace and Sawdust", *Hortsci.ashspublications.org*, 2018.

Joshi and D. Sandhu, "Preparation and evaluation of an animal feed byproduct produced by solid-state fermentation of apple pomace", *Bioresource Technology*, vol. 56, no. 2-3, pp. 251-255, 1996.

Joshi, V. K., and Neerja S. Rana. "Optimization of apple pomace based medium and fermentation conditions for pigment production by Sarcina sp." (2011).

Joshi, V. K., et al. "Microbial pigments." (2003).

Joshi, Vinod Kumar, Mukesh Parmar, and Neerja S. Rana. "Pectin Esterase Production from Apple Pomace in Solid-State and Submerged Fermentations." *Food Technology & Biotechnology* 44.2 (2006).

K. Babu, “α-Amylase Production by Thermophilic Bacillus coagulans in Solid State Fermentation,” *Process Biochemistry*, vol. 30, no. 4, pp. 305–309, 1995.

Leck, Astrid. "Preparation of lactophenol cotton blue slide mounts." *Community Eye Health* 12.30 (1999): 24.

M. Ramesh and B. Lonsane, "Solid state fermentation for production of higher titres of thermostable alpha-amylase with two peaks for pH optima byBacillus licheniformis M27", *Biotechnology Letters*, vol. 11, no. 1, pp. 49-52, 1989.

M. Stredansky, E. Conti, S. Stredanska and F. Zanetti, "γ-Linolenic acid production with Thamnidium elegans by solid-state fermentation on apple pomace", *Bioresource Technology*, vol. 73, no. 1, pp. 41-45, 2000.

N. Manoj Kumar, S. Karthikeyan and G. Jayaraman, "Thermostable alpha-amylase enzyme production from Bacillus laterosporus: Statistical optimization, purification and characterization", *Biocatalysis and Agricultural Biotechnology*, vol. 2, no. 1, pp. 38-44, 2013

R. Battaglino, M. Huergo, A. Pilosof and G. Bartholomai, "Culture requirements for the production of protease by Aspergillus oryzae in solid state fermentation", *Applied Microbiology and Biotechnology*, vol. 35, no. 3, 1991.

R. Shalini and D. Gupta, "Utilization of pomace from apple processing industries: a review", *Journal of Food Science and Technology*, vol. 47, no. 4, pp. 365-371, 2010.

S. Ramachandran, A. Patel, K. Nampoothiri, F. Francis, V. Nagy, G. Szakacs and A. Pandey, "Coconut oil cake—a potential raw material for the production of α-amylase", *Bioresource Technology*, vol. 93, no. 2, pp. 169-174, 2004.

S. Sivaramakrishnan, D. Gangadharan, K. Nampoothiri, C. Soccol and A. Pandey, "Alpha amylase production by Aspergillus oryzae employing solid-state fermentation", Nopr.niscair.res.in, 2018.

Sandhu, D. K., and V. K. Joshi. "Solid state fermentation of apple pomace for concomitant production of ethanol and animal feed." *Journal of scientific & industrial research* 56.2: 86-90,(1997).

S. Singh, S. Singh, V. Bali, L. Sharma, and J. Mangla, "Production of Fungal Amylases Using Cheap, Readily Available Agriresidues, for Potential Application in Textile Industry," *BioMed Research International*, vol. 2014, pp. 1–9, 2014.

V. Kalia, A. Kumar, S. Jain and A. Joshi, "Biomethanation of plant materials", *Bioresource Technology*, vol. 41, no. 3, pp. 209-212, 1992.

Villas-Bôas, Silas Granato, Elisa Esposito, and Margarida Matos de Mendonca. "Bioconversion of apple pomace into a nutritionally enriched substrate by Candida utilis and Pleurotus ostreatus." *World Journal of Microbiology and Biotechnology*19.5 (2003): 461-467.

Y. Hang and E. Woodams, "Solid state fermentation of apple pomace for citric acid production", *MIRCEN Journal of Applied Microbiology and Biotechnology*, vol. 2, no. 2, pp. 283-287, 1986.

Y. Lu and L. Foo, "Identification and quantification of major polyphenols in apple pomace", *Food Chemistry*, vol. 59, no. 2, pp. 187-194, 1997.

Y. Lu and L. Yeap Foo, "Antioxidant and radical scavenging activities of polyphenols from apple pomace", *Food Chemistry*, vol. 68, no. 1, pp. 81-85, 2000.