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Cellulase production by *Neurospora sitophila* utilizing press cake as substrate under solid -state fermentation

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Abstract

A fungal strain *Neurospora sitop*hila is analyzed to utilize Second generation lignocellulosic biomass press cake and brown juice under submerged and solid-state fermentation condition. Three main enzymes FPase, CMCase and β-glucosidase were studied in both conditions. The Solid-state fermentation condition is found most suitable for enzyme activity. Sterile and non-sterile media is also analyzed but heavy contamination is observed in non-sterile media. The different dilutions of brown juice with water were evaluated and (5%) Brown juice found supportive for microbial enzyme activity. Two different temperature were also studied and different agricultural residues such as (wheat bran, wheat grains, and Oat grains) were also analyzed in SSF condition. Wheat bran (1%) was found inducive for higher enzyme activity. Three different additional nutrients (Jaggery, Urea and Mustard oil) were also supplemented in SSF to find out their role as inducer but there was less enzyme secretion was performed by fungal strain. Finally, after optimizing some conditions SSF based tray bioreactor was designed in lab for batch operation. A notable increment in enzyme activity was observed.

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(AMIT SHARMA)

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1.Introduction

1.1 Background:

The scarcity of the ozone level in the upper atmosphere and persisting lethargic measurement and lack of common consensus among the nations has alarmed the entire world. Constant burning of fossil fuel (coal, oil, and natural gas) and land cleaning operations for acquiring more agricultural land in developing countries has profoundly increased the level of greenhouses gases. Continuous increment of greenhouse effects in earth environment is directly enhancing and causing the Earth warming. The recent sharp changes in environmental behavior across the world have stressed the scientific community to develop an ideal sustainable eco-friendly approach without affecting the environmental carbon equilibrium. Developed nations have shifted their focus from fossil-based fuels and products to bio-based fuels and other products. European Union has encouraged biorefineries heavily and currently more than 40 lignocellulosic biorefineries are under operation in Europe (Singh et al, 2019). The biobased economies have become a prime reality now to develop and construct a sustainable approach to replace the fossil-based platform. Biobased refineries can be a future solution to balance the environmental equilibrium in the production of fuels, chemicals, and other related products.

1.2 Biorefineries:

Biorefining is a sustainable process to exploit the available biomass into value-added products such as energy food, feed, materials, and chemicals (IEA Bioenergy, 2009). The definition of biorefinery term is to explore the technologies, and development of methods to exploit and convert raw biomass (lignocellulosic waste, agricultural residues, grains, wood, and municipal waste) into valuable building blocks useful for food, feed, energy, and value-added product formation.

A successful bio-economy is dependent on utilization of all possible available biomass economical bioconversion into energy, food, and valuable chemicals. A fully scalable and profitable bioconversion process to extract maximum sugar from available biomass is a current prime research agenda among the scientific community (Hassan et al, 2019). Presently day by day increasing population growth (83 million people each year) will touch 8.5 billion population on earth by 2030

(Dugarova and Gulasan ,2017). To fulfill and meet the huge demand for food, energy and other chemical products, we need a sustainable solution to satisfy our need without following conventional fossil-based processes. To keep the environmental sustainability in focus biorefinery is the best-suited solution in present scenario. European Bioeconomy strategy was launched in 2012 by all active Biorefinery industries (Hassan et al, 2019). The theme of such strategy was to the production of renewable biological resources and wastes into value-added products. This strategy and its action plans increased the turnover of the total economy in the European Union (EU) from €2.09 trillion in 2008 to 2.29 trillion in 2015 (Piotrowski 2018). There was 224 biorefineries operation across Europe in 2017 (Bio-based News, 2017). There were total 181 first generation biorefineries operating which use feedstock sugar, starch, oils, and fats and produce biofuels and related chemicals. However only 43 biorefineries known as second-generation biorefineries were under operation. These biorefineries convert lignocellulosic feedstock, nonfood, and nonenergy crops and biowaste into biofuel, electricity, heat, biobased chemicals and biomaterials (Hassan et al, 2019). The European Union has funded many projects under the scheme Horizon 2020 (~€80 billion research funding from 2014-2020) to utilize lignocellulosic feedstock under lignocellulosic biorefineries development in Europe (Table-1).

Project name	Biorefinery feedstock	Country coordinated in	Period	Total Cost (€)
AgriChemWhey	Byproducts from dairy processing	Ireland	2018-2021	29,949,323
GRACE	Miscanthus or hemp varieties from marginal lands	Germany	2017-2022	15,000,851.21
SmartLi	Kraft lignins, lignosulfonates, and bleaching effluents	Finland	2015-2019	2,407,461.25
BIOSKOH	Lignocellulosic feedstock	Italy	2016-2021	30,122,313.75
BARBARA	Agri and food waste	Spain	2017-2020	2,711,375
AgriMax	Agri and food waste	Spain	2016-2020	15,543,494.56

PULP2VALUE	Sugerbeet pulp	Netherlands	2015-2019	11,428,347.50
GreenSolRes	Lignocellulosic residues or wastes	Netherlands	2016-2020	10,609,637.01
Dendromasse4Europe	Dendromass on marginal land	Germany	2017-2022	20,442,318.75
SYLFEED	Wood residues	France	2017-2020	14,976,590
GreenProtein	Vegetable residues from packed salad processing	Netherlands	2016-2021	5,546,519.99
PROMINENT	Cereal processing side streams	Finland	2015-2018	3,103,897.50
FIRST2RUN	Cardoon from marginal lands	Italy	2015-2019	25,022,688.75
Zelcor	Lignocellulosic residues from ethanol production, lignins dissolved during pulping process, and lignin-like humins formed by sugar conversion	France	2016-2020	6,710,012.50
STAR4BBI	Lignocellulosic feedstocks from forsts and agriculture	Netherlands	2016-2019	995,877.50
BIOrescue	Wheat straw and agroindustrial waste	Spain	2016-2019	3,767,587.50
OPTISOCHEM	Residual wheat straw	France	2017-2021	16,376,816.83
US4GREENCHEM	Lignocellulosic feedstock	Germany	2015-2019	3,803,925

FUNGUSCHAIN	Mushroom (Agaricus bisporus) farming residues	Netherlands	2016-2020	8,143,661.25
POLYBIOSKIN	Food waste	Spain	2017-2020	4,058,359.38
ValChem	Woody feedstock	Finland	2015-2019	18,502,703.25
LIBBIO	Andes lupin from marginal lands	Iceland	2016-2020	4,923,750
LIGNOFLAG	Straw	Germany	2017-2022	34,969,215

Table-1: Lignocellulosic biorefineries development in Europe (Hassan et al, 2019)

In First generation biorefinery, Feed stock is edible fraction of food crops. the main product is biofuel. Second generation biorefinery based on lignocellulosic part of crops. This type of biorefinery converts nonfood part of plant, short rotation grasses that have high yield and easily cultivated on poor soils (poplar, willow, eucalyptus, alfalfa , reed canary, Napier and Bermuda and several agricultural residues (Bagasse , rice husk, rice bran, corn stover, wheat straw and bran etc) (Fig-1).

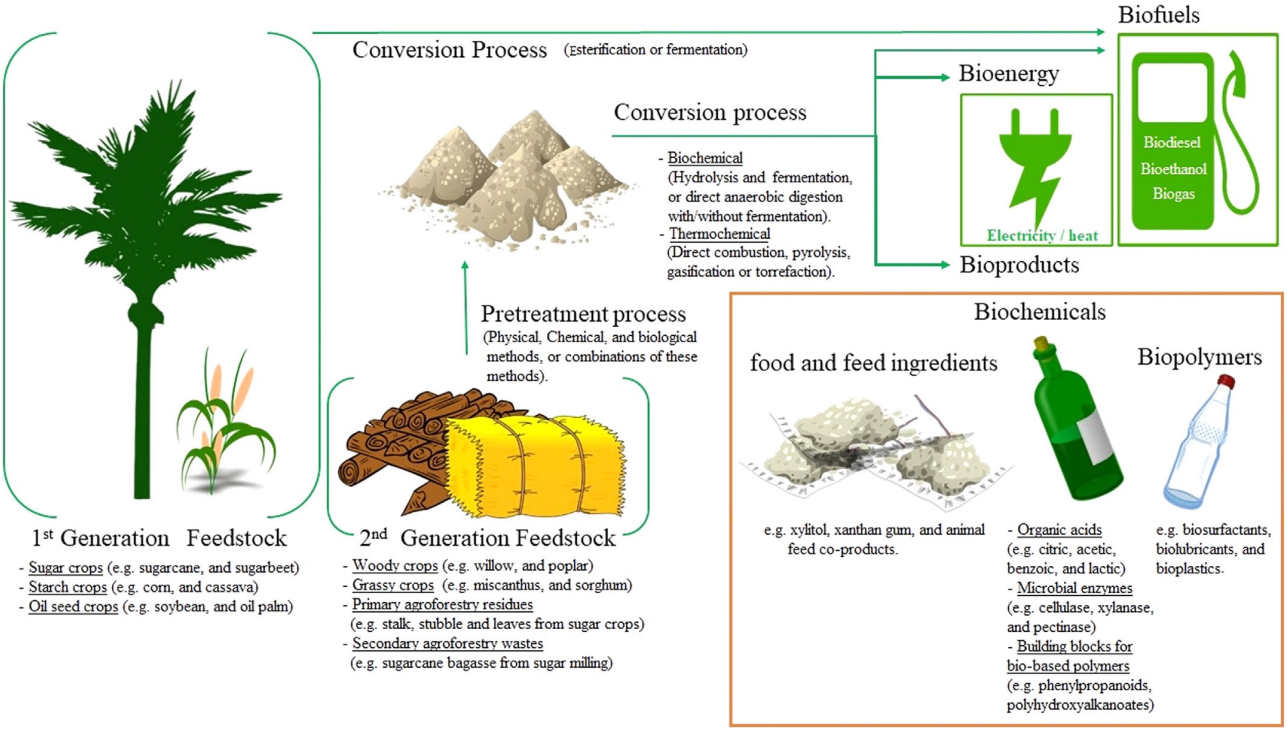


Figure-1: Schematic diagram difference between lignocellulosic feedstock from first and second generation sources and end products (Hasan et al, 2018)

1.3 Green Biorefinery:

The green biorefinery uses whole green crops, grass, clover, lucerne or alfalfa to produce energy, fuels, and value-added chemical. By exploiting the agricultural products and residues a sustainable method can be developed in Europe (Mandl, 2010). Green biorefineries are multiproduct system that performs and produce on the basis of physiology of raw plant material and using the diversity and availability by natural local conditions. A green biorefinery totally depends on sustainable raw material, technology, transportation, energy supply and land use (Digman et al 2013,).

The green biorefinery concept is based on a sustainable principle (sustainable use of land, raw material, technology, and energy). Production of proteins, fermentation media, animal feed, and biogas (Fig- 1.1) was obtained in green crop drying plant (capacity of 20,000 tons of alfalfa and green biomass) in Havelland, Germany (Kamm, 2013,Kamm Hille, Schonicke & Dautzenberg 2010).

A big disadvantage in green biorefinery is the availability of green biomass due to seasonal crops cultivation of grass depends on the vegetation period in Europe (April to October). This problem can be overcome by ensiling the green biomass and availability of raw biomass the be maintained in biorefineries all year. The green biorefinery is a reasonable way to use the grassland through a sustainable approach and preservation of cultural landscapes can be done (Kamm et al, 2016). In Germany green biomass is obtained from one-third of the total agricultural area of about 17 million hectares, which gives estimated at 6 million hectares resulted a yield of about 42 million tons of green biomass. In Europe total agricultural area covers 470 million hectares and one-third area is permanent grassland. This area is equal to 156 million hectares having the yield 1-billion-ton green biomass. While worldwide 4.9 billion hectares are used for agricultural cultivation, in this two-third area (3.3 billion hectares) is covered with green biomass (FAO stat, 2012). Alfalfa is most important forage crop due to high nitrogen absorbing ability from air. Alfalfa globally cultivated on about 30 million hectares in which around 25% (7.2 million hectares) obtained from Europe (Cash, 2009).

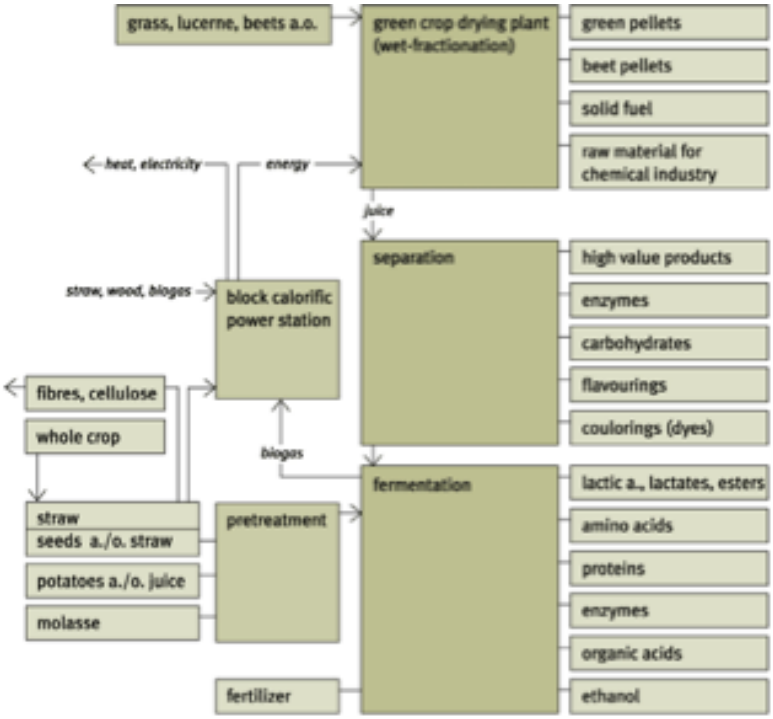


Figure-1.1: Green biorefinery for the manufacture of food and non-food products (Kamm et al, 2016)

1.4 Organofinery project:

Recently an esteemed organofinery project is under operation. The project is under the supervision of Mette Lubeck and the aim of this project is to generate energy (bio), fertilizers and pure organic proteins for monogastric animals. (Fig-1.2) depicts an outline of this project.

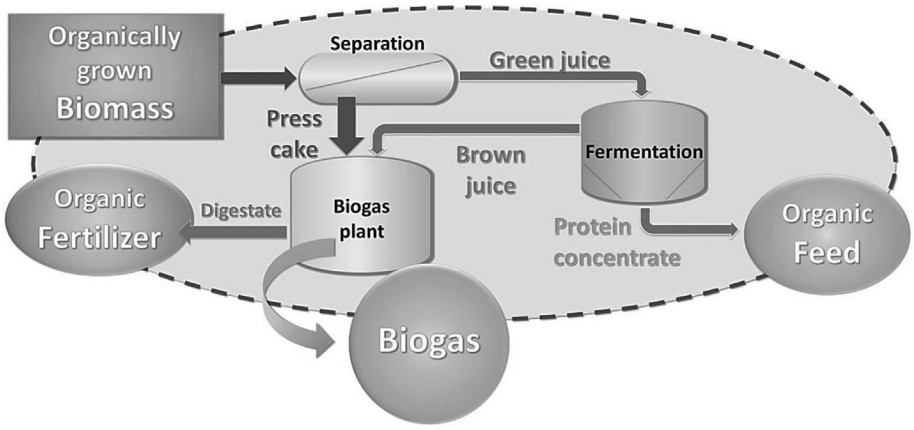
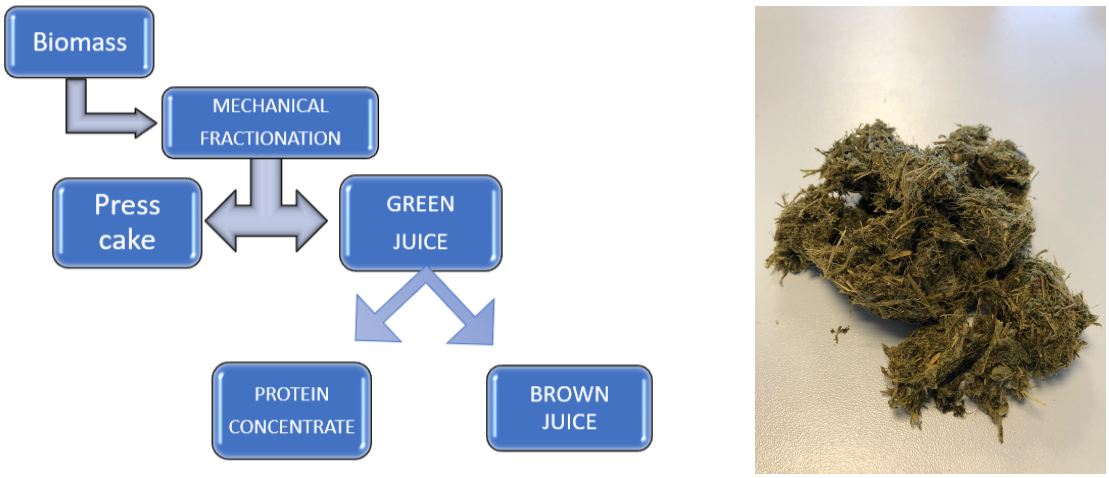


Figure-1.2: Scheme of organofinery project (Santamarie Fernandez M. S. et al. 2018)

There are several plants have been evaluated but mainly plants belong to family Fabaceae (Leguminosae) popular as legumes have proved best suited for the task. These plants contain root nodules in which nitrogen-fixing bacteria Rhizobium lives under symbiosis in root nodules. These gram-negative soil bacteria have the ability to fix the nitrogen for plant. The legumes evaluated in project is mainly alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L), clover grass (*Trifolium* sp.) and the crucifer oilseed radish (*Raphanus sativus* L.)

1.5 Press cake and brown juice:

There are two steps to obtain brown juice and press cake from grass (Fig-1.3). The first step is a mechanical fractionation of grass giving two main fractions. The main greener solid part called press cake. Presse cake contains lower crude protein, ash and ether extract but a higher amount of crude fiber, lignin, and cellulose (Walker et al., 1982). While the second obtained liquid fraction called green juice. The green juice has good nutritional value and it contains good amount of proteins and other nutrients, the valuable components can be utilized through lactic acid fermentation. After lactic acid fermentation, the remaining byproduct is called brown juice (Santamarie Fernandez M. S. et al. 2017*)*. Press cake consists mainly of lignocellulosic material and it is considered as an agriculture residue. Brown juice is comprised of several nutrients such as sugars, amino acids, organic acids, and minerals (Santamarie Fernandez M. S. et al. 2017*)*.



(a) *(b)*

Figure-1.3(a): Press cake and Brown juice separation, (b) Press cake

1.6 Lignocellulosic Biomass :

A typical plants cell wall is made up of lignocellulosic materials containing cellulose, hemicellulose, and lignin (Fan et al, 2016). Both cellulose and hemicellulose are comprised of polymers sugars, while on the other hand, lignin is composed of complex aromatic polymer made from phenylpropane units (Fig- 1.4). Lignocellulosic content and composition in the plant vary on the basis of different species (Perez et al, 2002).

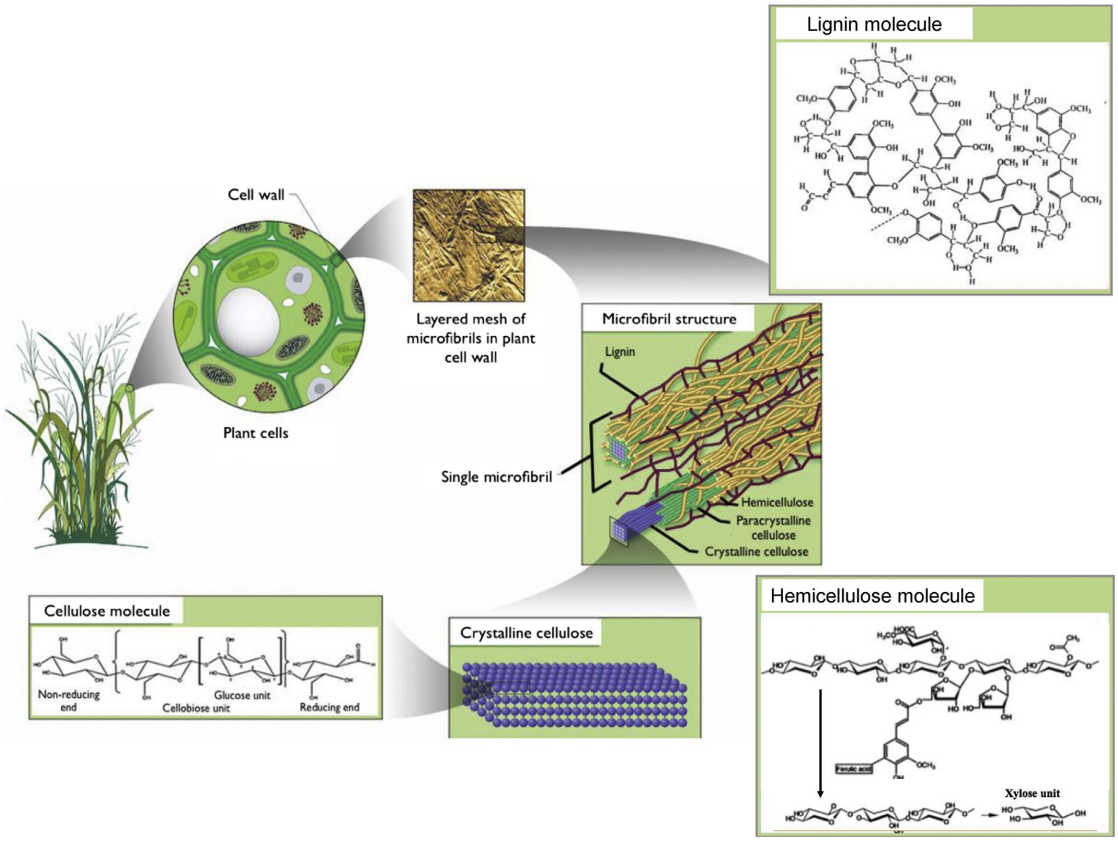


Figure-1.4: Lignocellulosic structure (Seidl et al.,2016)

Cellulose is one of the most important polymers in the cell wall. It is composed of sugar D-glucose which is linked by β-1,4 glycosidic bonds. The β linkage bonding in cellulose results into a long chain attached together by hydrogen bonds and van der Waals forces (Perez et al, 2002).Cellulose in structural form has two different regions, The amorphous region is part of cellulose which has loose structure (Figure 1.5). The amorphous part of cellulose structure can be easily hydrolyzed through various pretreatments and different enzymatic hydrolysis carried out in bioconversion processes. The

crystalline region of cellulose is quite rigid and strong in comparison to the amorphous region. It is composed systematic and arranged structure. This compact and strongly bonded region is very strong and difficult to hydrolyze by different enzymes and pretreatment process (Perez et al, 2002).

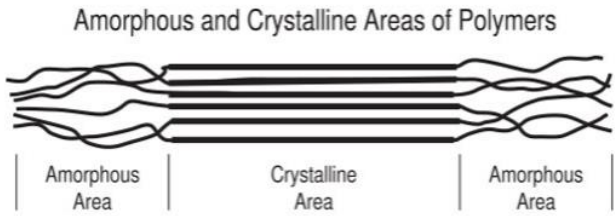


Figure-1.5: Amorphous and Crystalline regions in cellulose

Hemicellulose is a sugar polymer containing both six-carbon sugars and five-carbon sugars. The six-carbon sugars consist of glucose, mannose, galactose, whereas the five-carbon sugars include xylose and arabinose (Perez et al, 2002). These sugars are linked together by two types of linkages, β-1,4, and β-1,3-glycosidic linkages. Hemicellulose differs from cellulose, in that it is an amorphous and heterogeneous molecule that can almost be degraded in pre-treatment process (Fan et al, 2016).

Lignin is an aromatic polymer, composed of three phenylpropane units: sinapyl alcohol, trans-p-coumaryl and coniferyl alcohol (Fig- 1.6) (Perez et al, 2002). It is an amorphous and heteropolymer, which is attached to cellulose and hemicellulose forming an inaccessible region in the plant cell wall. Lignin is characterized by giving the plant cell wall the structural support and by its non-hydrolysable linkages, which give the resistance against microbial access.

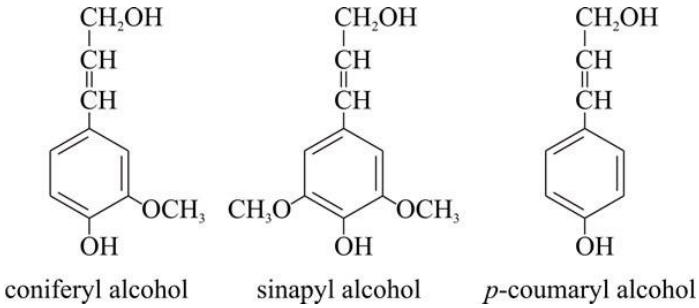


Figure -1.6: *The three phenyl propane monomers in lignin*.

1.7 Cellulase :

Cellulase is a hydrolytic enzyme that induces hydrolysis of beta linkages in cellulose. The cellulase enzyme complex contains three different kinds of the enzyme that act together to degrade cellulose. Full hydrolysis of cellulose requires a complex synergy among different cellulolytic enzymes, including endoglucanases, Cellobiohydrolase (exoglucanases) and β- glucosidases (Fig- 1.7). Endoglucanases initiate the degradation of cellulose by attacking the amorphous region in the cellulose which results in free ends. Then Cellobiohydrolases bind to the free ends and release dimer called cellobiose. Finally β-glucosidases liberates glucose by degrading cellobiose (Saqib et al.,2010).

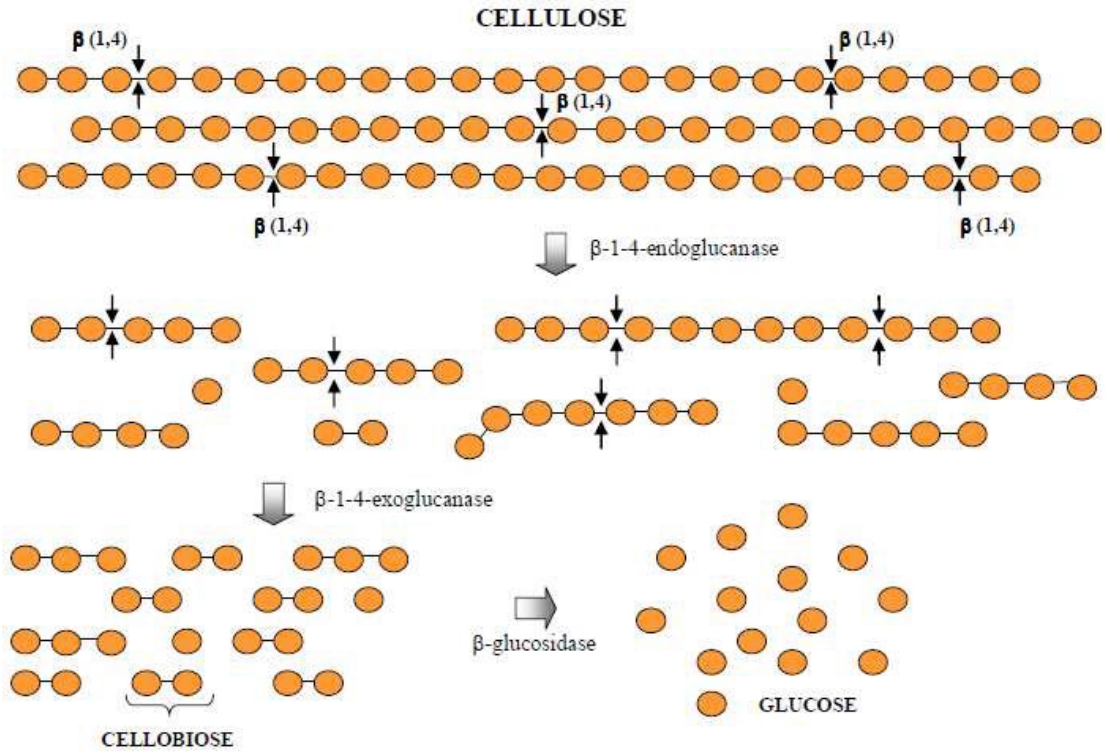


Figure 1.7 : Enzymatic hydrolysis of cellulose by synergistic action of cellulases (Mussatto and Teixeria, 2010)

1.8 Fungal Biocatalyst:

Lignocellulose is the most abundant carbon source present on earth (Gupta et al., 2016). Microorganisms such as white, brown and soft-rot fungi are capable of degrading such biomass. While among them white-rot fungi are the most potent due to their unique ligninolytic system (Eriksson et al.,1990). Fungi play a large and prominent role in valorization of lignocellulosic biomass. Fungal organisms are the best choice because firstly, they produce plant cell wall degrading enzymes that aid in the decomposition of biomass by hydrolyzing the lignocellulosic polymers. Secondly, they produce various value-added products and important building blocks through biochemically during fermentation process. Nowadays an attractive idea has surfaced to produce protein-rich fungal biomass by utilizing low-value lignocellulosic biomass. Biodegradation of lignocellulosic biomass through fungal bioconversion produces fungal biomass along with desirable products. Protein-rich fungal biomass of *Rhizopus microsporus* for aquaculture feed was produced by utilizing waste from ethanol industries (Nitayavardhana et al.,2010).

1.9 *Neurospora sitophila*:

In the present study, Neurospora sitophila is selected to utilize press cake for cellulase enzyme production studies. Fungal strain *Neurospora sitophila* is from phylum Ascomycota. This is very fast-growing fungi and has off-white color mycelia and orange color spores (Fig- 1.8). This is an obligate aerobes fungus and highly reproductive and has extensive uses in industries. *Neurospora sitophila* has lot of importance in Asian food preparation. It is used traditional Indonesian dish oncom. Fungal spores and fast mycelial growth cause huge contamination in bakery operation. This fungal strain has uses in food industries and considered as GRAS.

Fig- 1.8 N.sitophila growth in nature and microscopical view of conidia. (©-*Neurospora sitophila* Sear & B.O. Dodge (JEN 309)., Joan Carles Salom)

1.10 Solid-state Fermentation Process:

Solid-state fermentation (SSF) is defined as the fermentation involving solid in the absence (or near absence) of free water, however, the substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey et al., 2000a). SSF has gained importance due to several advantages over submerged fermentation, such as superior productivity, simplicity, reduced energy requirements, low wastewater output, and improved product recovery. In recent years SSF has been exploited for the production of a spectrum of value-added products e.g. antibiotics, alkaloids, plant growth factors, biofuel, enzymes, organic acid, and aroma compounds.

Generally, substrates used for SSF are of agricultural origin. In recent years, there has been a spurt in the activities towards utilization of agro-based residues such as cassava bagasse (Pandey et al., 2000b), sugar cane bagasse (Pandey et al., 2000c; Kumar et al., 2003), wheat and the rice straw, (Laukevics et al., 1984, Suhartono et al., 1991) etc. for SSF process. However, some groups had also employed fruit wastes for organic acid production (Kumar et al., 2003b; Roukas, 2000).

Moisture content during SSF is a very crucial factor that affects the microbial growth and product formation. Higher moisture levels may displace gases from the interparticle spaces and may cause agglomeration of substrate particle into lumps (Kim et al., 1985; Ellaiah et al., 2004). Whereas, low moisture level reduces substrate availability to the microorganism which hinders the product formation (Pandey et al., 2000a; Kumar et al., 2003; Ellaiah et al., 2004). Besides above, maintenance of the desired oxygen level during process may be another pre-requisite during SSF process.

Optimal airflow during SSF may be based on the oxygen dependence of the process for product synthesis. Further, removal of heat generated during process, mass, thickness and the inherent air

spaces of the substrate are the other factors deciding the oxygenation levels required during the process (Lonsane et al., 1985; Kalogeris et al., 2003).

1.11 Tray bioreactor :

Tray bioreactor consists of a chamber in which air with controlled temperature and relative humidity are circulated around a number of trays. Tray bioreactors have no forced aeration or mixing for the substrate. Each tray contains a thin layer of substrate typically between 5 to 15 cm deep and usually has an open-top (Mitchell et al., 2000). Such bioreactors are restrictive in the amount of substrate that can be fermented, as only thin layers can be used in order to avoid overheating and to maintain aerobic conditions. Glucoamylase and alkaline protease production were accomplished using the tray bioreactor (Ghildyal et al., 1994 and Malathi and Chakraborty, 1991). Recently agro-industrial wastes were utilized to produce pectinase enzyme SSF based tray bioreactor (Mahmoodi et al., 2019).

1.12 Project Objective and outlines:

There are following outlines of the present project

a) Evaluation of Press cake as cheaper substrate under submerged and solid-state fermentation by employing brown juice as another nutrient.
b) Study of *Neurospora sitophila* and its main cellulase enzyme activity in the fermentation process.
c) Optimization of solid-state fermentation condition under flask condition by analyzing temperature, agricultural residues, additional nutrients, and solid supports.
d) Designing of SSF based tray bioreactor under derived optimal conditions
e) Cellulase enzyme study in tray bioreactor operation

2. Material and Methods:

2.1 Microorganism:

Neurospora sitophila Shear et Dodge (NRRL 2884) was obtained from the lab of Aalborg University Section of Sustainable Biotechnology. The strain was grown on PDA medium at 30^0C temperature. Fungal strain also maintained PDA slants. The slants were maintained at 4^0C in lab.

2.2 Inoculum Preparation:

Five days old fully sporulated plates were used to make inoculant preparation. Spores were harvest with the sterile needle and suspended in sterile water containing 0.1% Tween 80. The spores concentration was adjusted by hemocytometer counting.

2.3 Fermentation Media:

The fermentation media used in both submerged and SSF fermentation was water, diluted brown juice and minimal media. The minimal for enzyme production contains (g/l) 10.0; $(NH_4)_2SO_4$, 1.70; urea, 0.516; KH_2PO_4, 2.0; $MgSO_4$, 0.2; $CaCl_2$, 0.2; $FeCl_3$, 0.16; $ZnSO_4$, 0.22; H_3BO_3, 0.006; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.024, $CuSO_4$, 0.039 and $MnCl_2$ 0.007 (Vlach and Young, 1992).

2.4 Press Cake and brown juice:

Press cake and brown juice obtained from lab of Aalborg University Section of Sustainable Biotechnology The press cake and the brown juice were stored at -18 ^{o}C for preservation. The dry mass of press cake was estimated by equipment (Mettler Toledo) and 0.712mg dry mass obtained from 2000mg PC. Hence, 64,4% of water content was reported in PC.

2.5 Submerged Fermentation:

Submerged fermentation was carried out in 50 ml of fermentation medium in Erlenmeyer flasks (250ml). The medium was inoculated and incubated at 30^0C in an orbital incubator shaker at constant shaking (180 rpm). The fermentation was carried out for 7 days. The fermented liquid sample (200 µL) was collected on each day under an aseptic condition in laminar airflow.

2.6 Solid-state Fermentation:

Solid-state fermentation was carried out by utilizing press cake (10gms) in Erlenmeyer flasks (250ml). Moisture content 24 ml (Water, diluted brown juice and minimal media) was added to moisten the press cake. The flasks were autoclaved at 120^0C for 20 minutes and inoculated 1 ml of inoculum having $4*10^7$ spores/ml. One set of unautoclaved flask was also inoculated in similar manner. The inoculated flask was kept at 30^0C temperature in incubator for 7 days' time period. The fermented liquid sample (200 µL) was collected on each day carefully under aseptic condition in laminar. A gentle partial shaking was also carried out carefully without disturbing or breaking layer of mycelial growth over the surface of fermentation media. Single flask was taken due to small incubation space and limited glassware.

Solid support and agricultural residues:

Fig 2.1: Oat, Wheat Bran and Wheat grains

Jute net and cotton fabric were procured from India while wheat bran, oat and wheat grains were collected from Lantmanen Cerealia, Vejle (Fig 2.1).

2.7 Design of Tray Bioreactor:

A tray bioreactor was designed in a plastic box (34x25x16cm) (8L) for batch production of cellulase enzymes as shown in (Fig 2.3). Two Rectangular plastic trays of the of 2 cm depths were horizontally arranged in parallel in the bioreactor (Fig- 2.2). To maintain aerobic conditions, sterile air was circulated through air pump unit over the bed surface. A humidifier tray filled with sterilized water was fixed in the bottom for maintaining humidity in the system. For solid-state fermentation in the

bioreactor, each tray was layered with solid support (100g, Press cake mixed with (1%) Wheat bran) which was then soaked and moistened with fermentation medium. Following inoculation, the microbial agent was allowed to grow over the support.

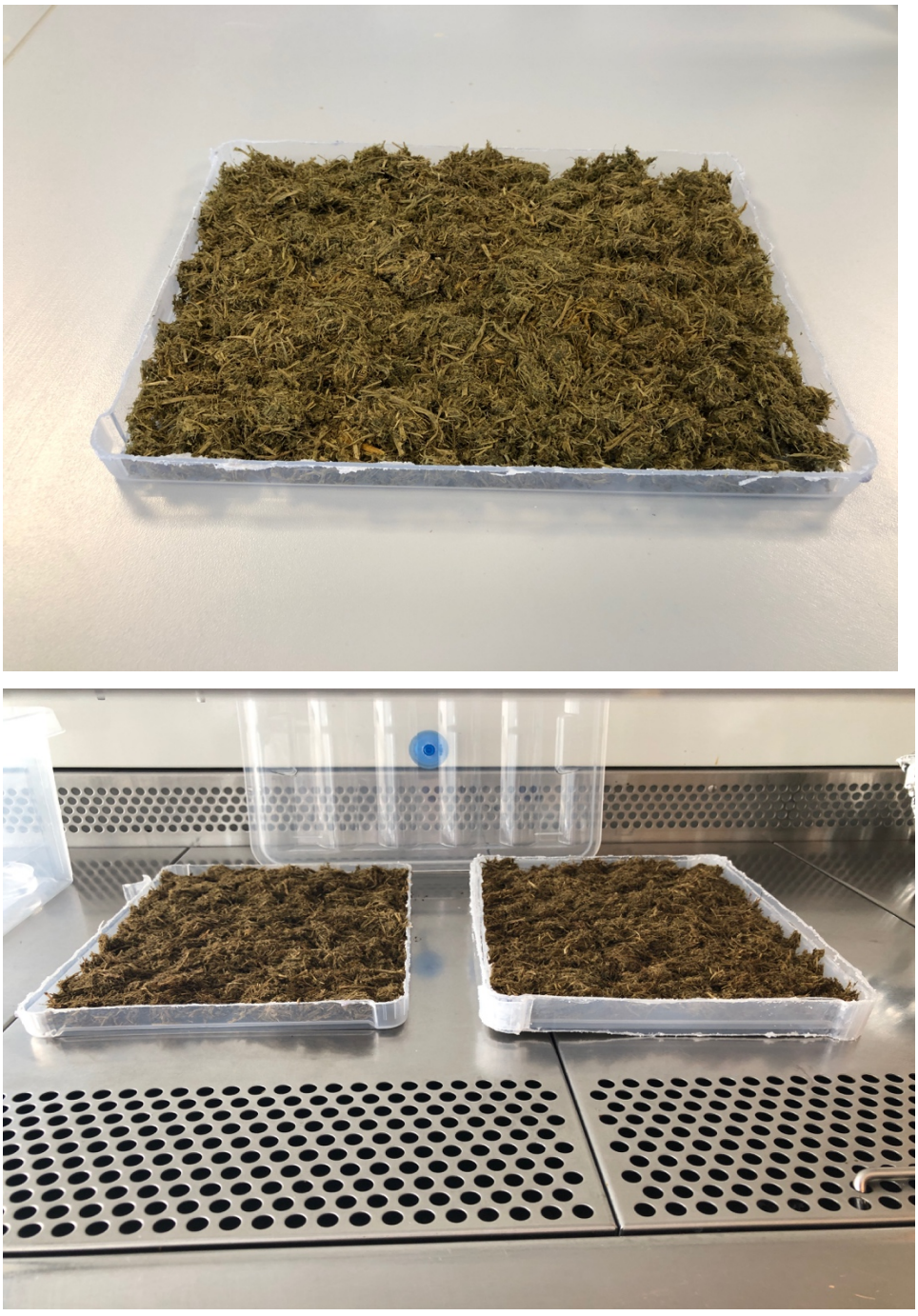


Fig 2.2: Trays filled with sterile press cake before inoculation

Fig 2.3: Tray Bioreactor aeration process

Fermented liquid sample (200 µL) was collected on every day of bioreaction operation in aseptic condition. and aeration was also performed by passing air through 20µ filter by a fitted air pump. Aeration was performed after every 24 h for 15 minutes on each tray. After completion of the fermentation cycle on 7th days, trays were subjected to UV sterilization to inactivate the produced spores on mycelial biomass, and then the tray content was squeezed to release the remaining accumulated enzyme. The fermentation broth recovered was used for estimating the enzyme production Three sample sets were taken with standard deviation.

2.8 Enzyme assay:

FPase assay:

The assay solution was first produced by the following ways: 0.5 mL of 5M sodium citrate was diluted up to 50 mL water, to get a concentration of 0.05 M. The buffer was then adjusted to pH 5.0. DNS reagent was produced by dissolving 10 g of DNS in 500 mL water, then 200 mL 2M Natrium hydroxide, and 300 g potassium sodium tartrate were added to the solution. After the salt was completely dissolved, water was added to a total volume of 1000 mL. The reaction started by adding 40 µL of the enzyme to 80 µL 0.05 M sodium citrate buffer in a 1.5 ml Eppendorf tube, then the tubes were incubated at 50 ^{O}C for 60 min. After 60 min the incubation was stopped by putting the samples on ice, then adding 240 µL of DNS substrate, followed by another incubation at 100 ^{O}C for 5 minutes. Then the samples were again put on ice, and finally 1000 µL of water was added. The samples were measured at an absorbance of 550 nm.

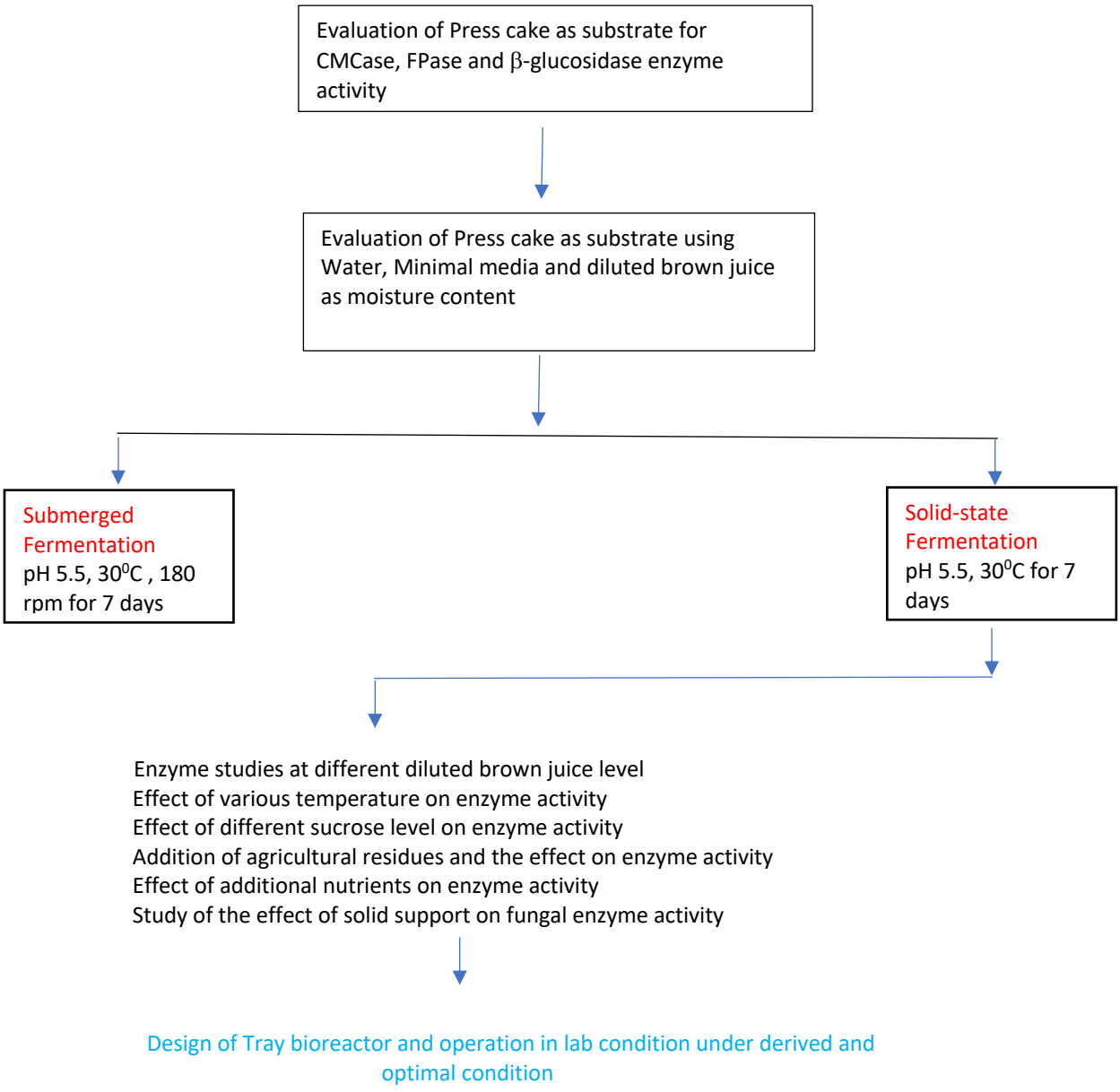
CMCase assay:

The CMCase enzyme is estimated by using dyed CM cellulose in following ways. 1 g dyed substrate was mixed with 40ml demineralized water and the homologous solution was made by stirring. Sodium acetate buffer 2.5 ml (2M, pH 4,5) was added and 50 ml volume was kept having pH 4,5. A precipitant solution was made by dissolving 20 gm of sodium acetate trihydrate and 2 g of zinc acetate in 75 ml demineralized water. The pH was maintained 5 and volume was made 100 ml. Ethanol(96%) 400ml was added in above prepared solution carefully with good mixing and stored at room temperature. The enzyme sample (125µl) was added with substrate (125µl) and incubated for 30 min, 40 ^{O}C at 700 rpm. The reaction was terminated by addition of 625µl precipitant solution with on vertex for 10 seconds. The reaction was allowed for 10 min and centrifuged for 10000 rpm for 10 min. The 100µl of supernatant was measured at 540 nm. The standard curve was also made Cellulase enzyme (Aspergillus niger).

Cellobiase assay:

The assay was performed by adding 15 mM cellobiose substrate into 0.05 M citrate buffer and pH was adjusted 4.8. Enzyme β- glucosidase activity was measured by microtiter plates. Substrate mix (50µl) was taken in an Eppendorf tube at 50^0C temperature on thermoshaker. Enzyme preparation (5µl) was added and mixed well and kept for 30 minutes. After incubation (30µl) mixture was placed microtiter plates well and (50µl) stop reagent was added to stop the reaction and absorbance was measured at 405nm by spectrophotometer Cellobiose blank and enzyme blank were also measured.

2.9 Flow diagram of work done:



3. Results and Discussion:

3.1 Morphological features of Fungi:

The characteristic and morphological features of the wild type fungal strains were observed. The hyphae of wild type *Neurospora sitophi*la were thinner and elongated (Fig.-3.1) The extent of sporulation was higher in fungal strain as compared to other common fungal strain. Further, spores of the fungi had uneven and rough surface and orange in color. The fungal growth was observed very fast and thicker and dense.

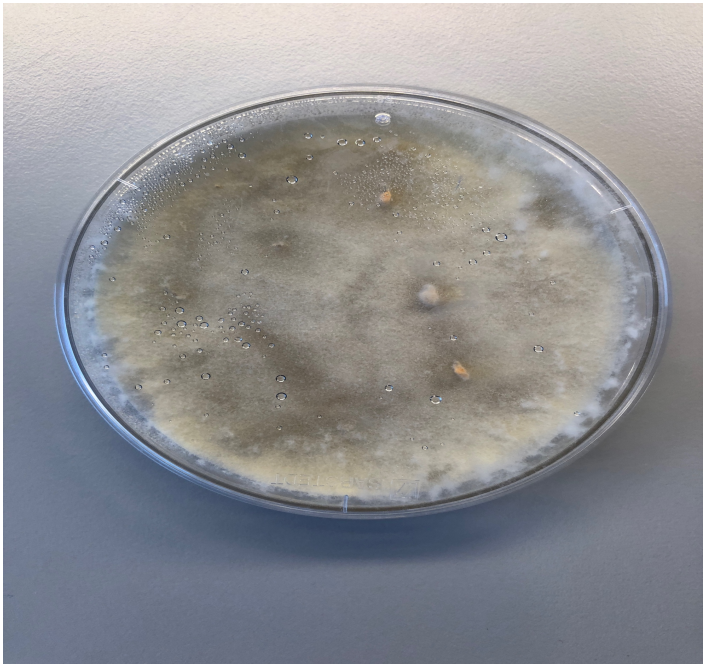


Fig-3.1 : *Neurospora sitophila* fungal growth (72h)

3.2 Evaluation of Press Cake under Submerged Fermentation:

The procured press cake is analyzed by employing preoptimized parameter under submerged fermentation condition at pH 5.5, Temp 30^0C and 180 rpm. The inoculated flasks were incubated for 7 days in a rotatory shaker. The enzyme activity was observed every day and results were shown in (Fig-3.2). Maximum FPAase and CMCase (0.024 Uml^{-1} and 0.018 Uml^{-1}) activity were observed on 3rd day. While maximal β-glucosidase activity was found on 5th days of incubation and a sharp decrease was observed after 5th days. There was no enzyme activity was observed in unautoclaved flask and contamination was clearly seen after 3rd days during visible observation of flasks.

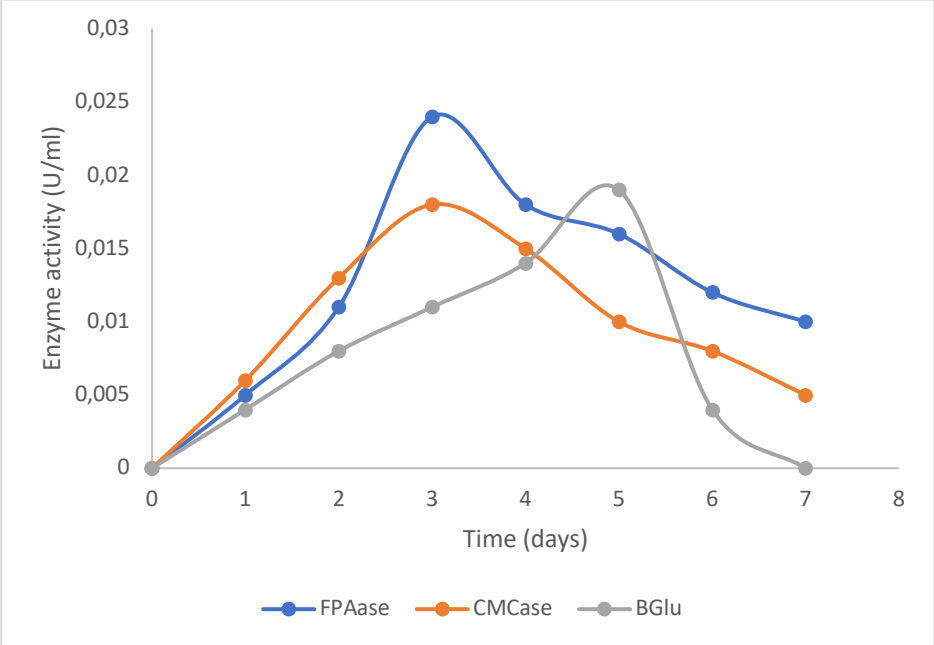


Fig-3.2: Press Cake with Minimal media under Submerged Fermentation

Evaluation of press cake was also carried out with water and diluted brown juice with above-preoptimized parameters but enzyme activity almost negligible (result not included) and it was noted that only minimal media was favorable for enzyme activity by the fungal strain.

3.3 Evaluation of Press Cake under Solid-state Fermentation:

Solid-state fermentation (SSF) condition was provided to microbial agent by using Press Cake as substrate and water, diluted brown juice and minimal media were analyzed as moisture content and flasks were incubated at 30^0C temperature with partial shaking during after every 24 hours. SSF was found to be much productive in term of enzyme activity in compare to submerged fermentation.

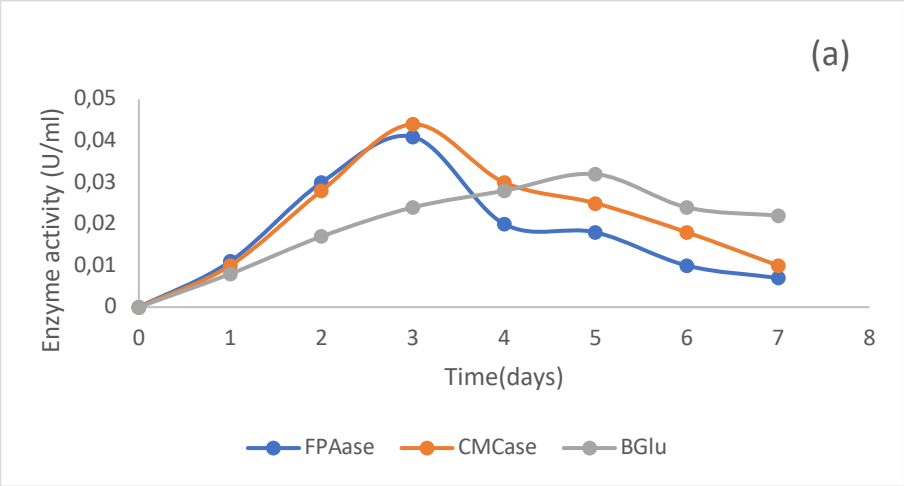


Fig-3.3(a): Enzyme activity using press cake and water under solid state fermentation

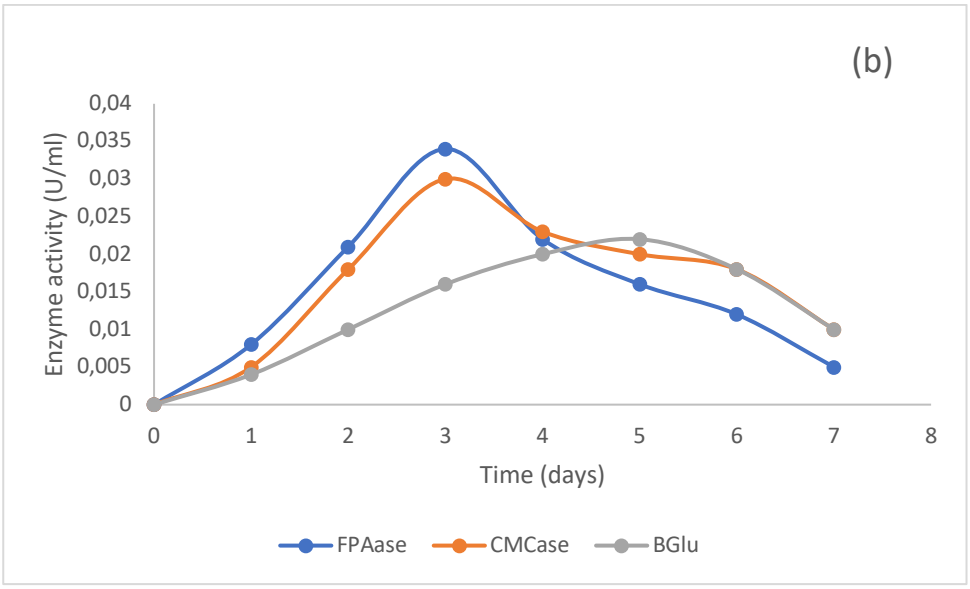


Fig-3.3(b): Enzyme activity using press cake and brown juice (2%) under solid-state fermentation

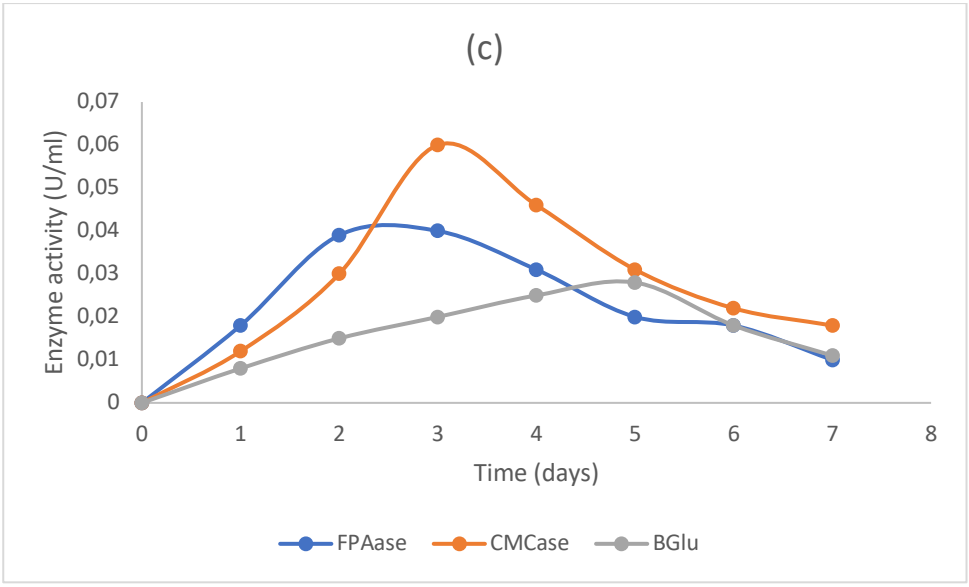
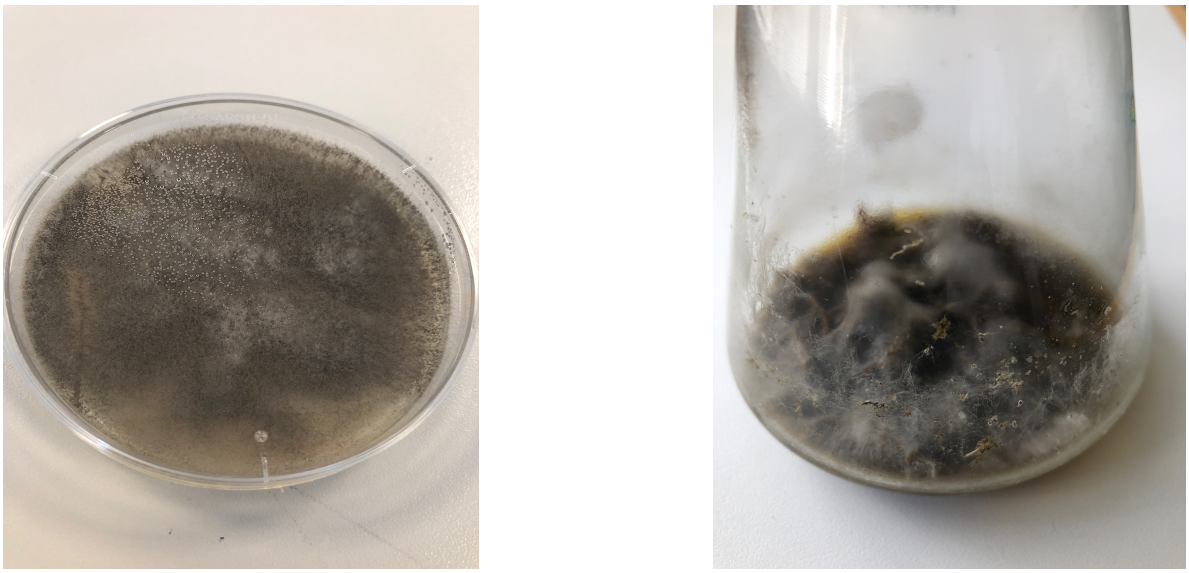


Fig-3.3(c): Enzyme activity using press cake and minimal media under solid-state fermentation

According to (Fig-3.3 a-c) the maximal enzyme activity was observed with press cake with minimal media in SSF condition. FPase activity 0.04 Uml^{-1} and CMCase activity 0.06 Uml^{-1} was observed on 3rd day of fermentation. While higher β-glucosidase activity 0.032 Uml^{-1} was reported on 5th days of fermentation when water was used as moisture content. On the other hand, lower enzyme activity of all three enzymes was observed with water and brown juice (2%) in SSF condition. Unautoclaved set of flasks for above set was also inoculated with fungal strain but clear contamination was found after 3rd days of fermentation.

The aim of this project was to establish an economical bioconversion process. Hence, unautoclaved fermentation processes were carried out to save energy used for sterilization process. Unfortunately, contamination was observed in all flask despite less water in SSF condition. The contaminant was quite dominative, but it was not inhibitory for *Neurospora sitaphila* fungal strain. The contaminant was isolated and purified and observed on PDA plates. Based on growth pattern and morphological study under light microscope Aspergillus niger was found as contaminant (Fig-3.4).



(a) (b)

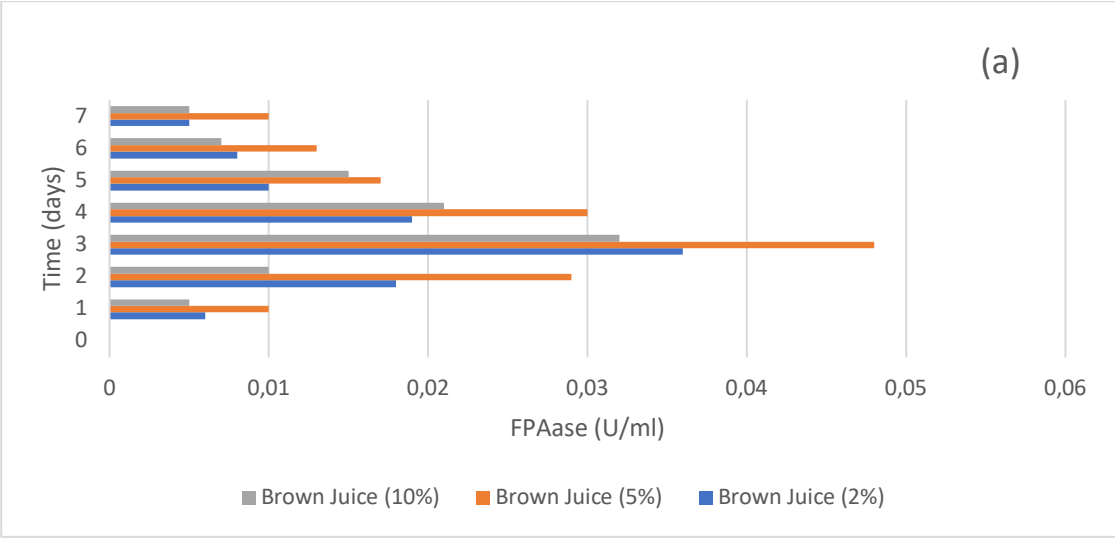
Fig: 3.4 (a) Aspergillus niger on PDA (84h)

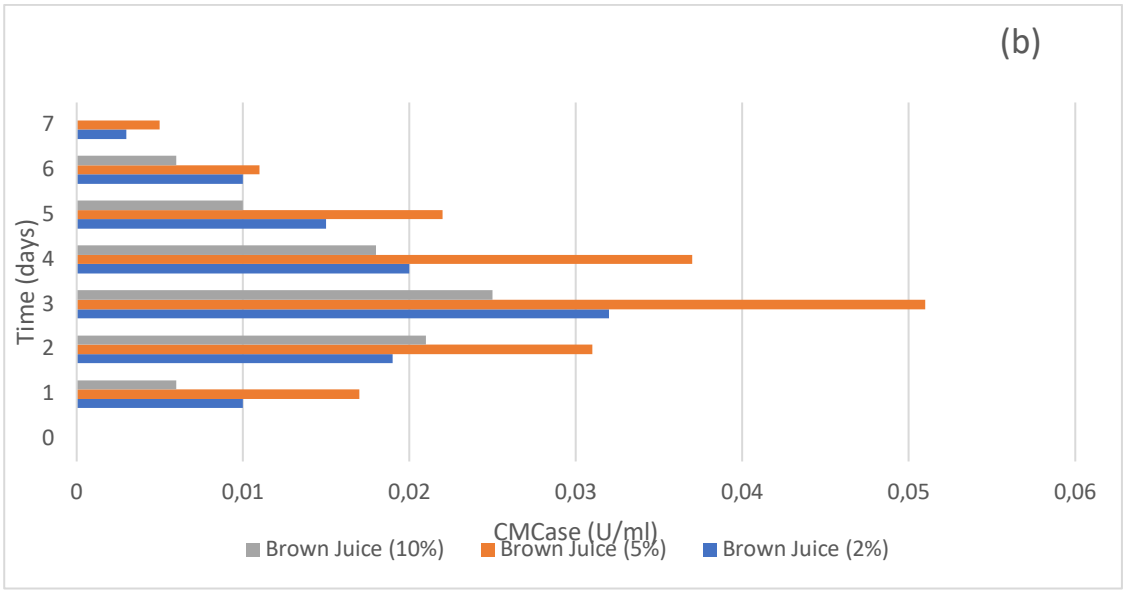
(b) Aspergillus niger contamination in the flask.

Solid-state fermentation is observed as best suited condition for notable enzyme activity by above results in compare to submerged fermentation. SSF is further evaluated and optimized for obtaining enhanced enzyme activity.

3.4 Effect of different level of brown juice as moisture content on enzyme activity in SSF:

Various level of brown juice (2,5 and 10%) diluted with water was analyzed in SSF condition. Press cake (10 gms) was moistened with 24 ml of diluted brown juice with water. The autoclaved flask is inoculated with measured fungal spores inoculum. SSF was carried out and results are represented in (Fig-3.5). According to results obtained brown juice (5%) is found encouraging for improved enzyme activity. Maximum FPase (0.048 Uml^{-1}) and CMCase (0.051 Uml^{-1}) is observed during the 3rd day of fermentation cycle. However, a pattern of decreased enzyme activity is clearly visible in results obtained. The β-glucosidase enzyme is produced higher (0,026 Uml^{-1}) during the 4th day of fermentation by the fungal strain.





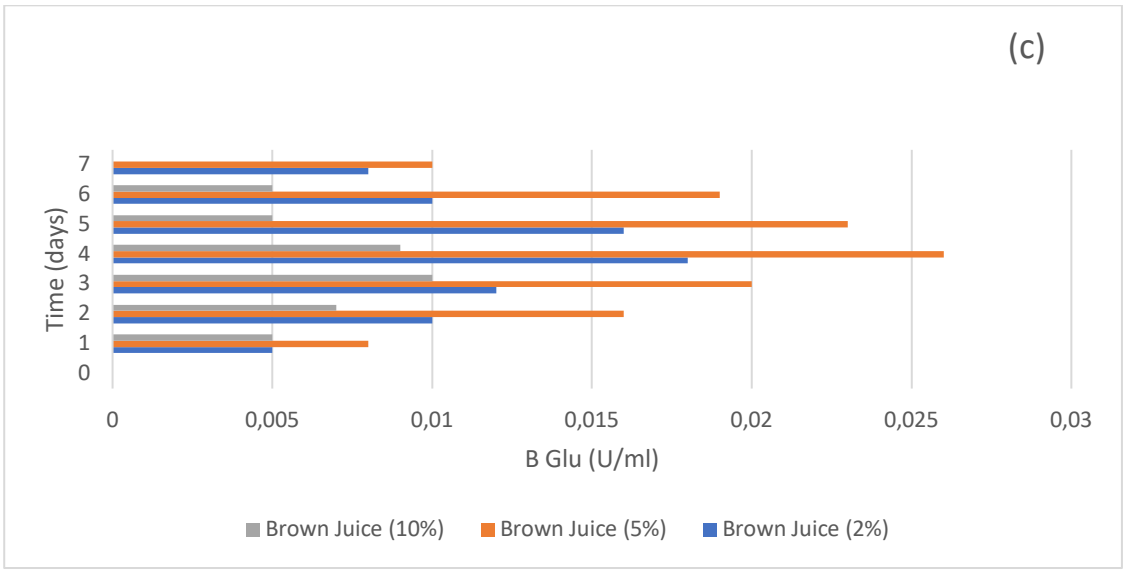


Fig -3.5 : Enzyme activity at different brown juice level under SSF condition

Analysis of enzyme activity as a function of temperature under SSF condition:

Effect of incubation temperature on *Neurospora sitophila* fungal strain in solid-state fermentation was is studied by incubating inoculated flask containing diluted brown juice moistened press cake. Temperature level 30 and 37^0 C were adjusted in incubation. Temperature 30^0 C is reported promising with maximal enzyme activity in compare to 37^0 C temperature incubation. In (Fig-3.6) the

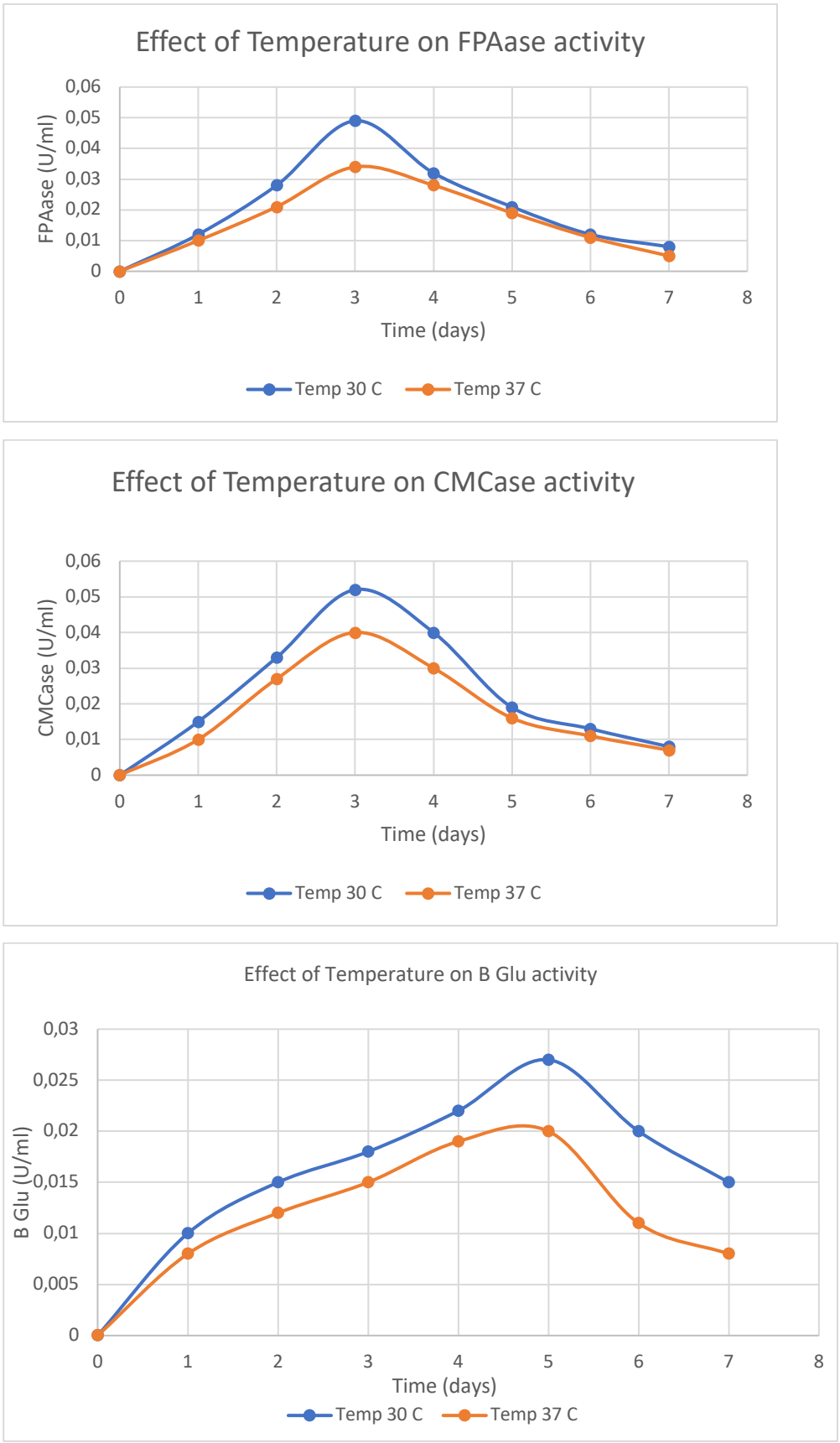
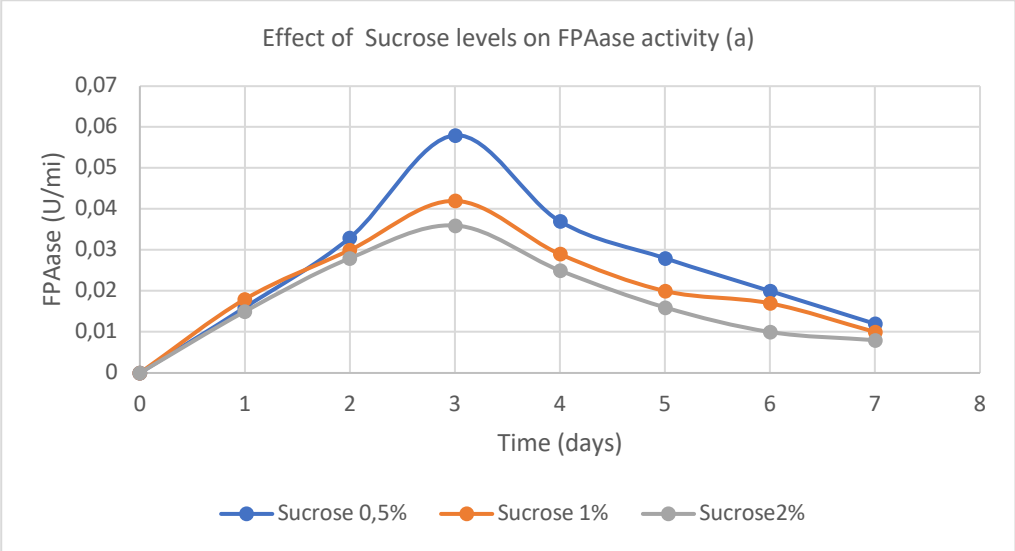


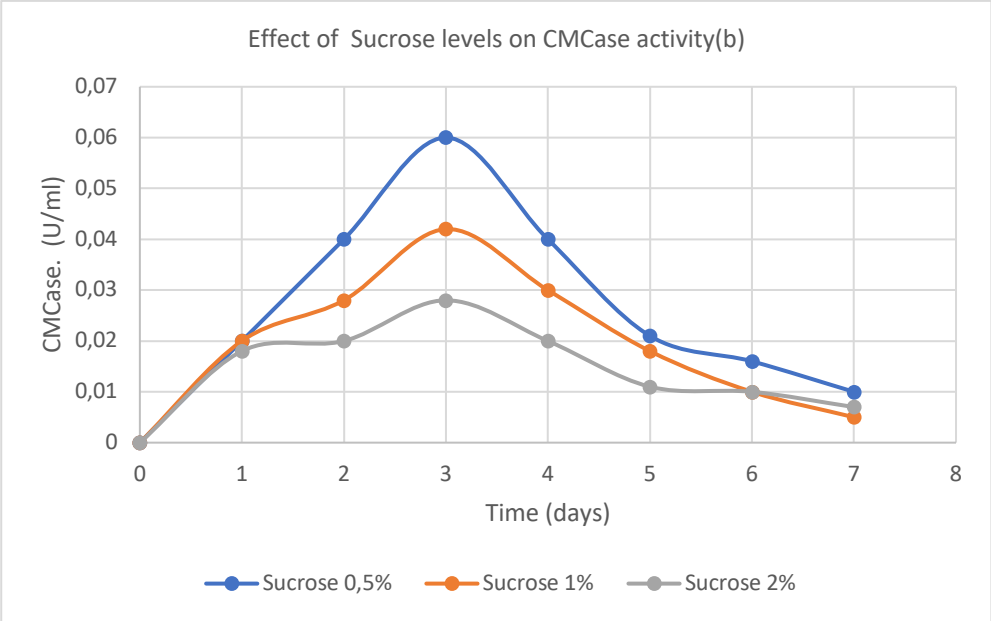
Fig-3.6: Effect of Temperature on Enzyme activity under SSF condition

The temperature level of 30^0C is found optimal for fungal strain for higher enzyme activity (Fig-3.6). Maximal FPase (0.049 Uml^{-1}) and CMCase (0.052 Uml^{-1}) activity are observed during 3^{rd} days in fermentation. Higher β-glucosidase enzyme activity (0.027 Uml^{-1}) found on 5^{th} days of Solid-state fermentation process. Later on β-glucosidase enzyme activity reduced slightly until 7^{th} days of fermentation cycle.

3.5 Effect of different level of sucrose on enzyme activity in SSF:

Supplementation of different sucrose level (0.5,1 and 2%) was analyzed by adopting previously obtained optimal conditions. A notable enhancement of FPAase enzyme activity is observed in the flask containing 0.5% of Sucrose with fermentation media. Higher FPAase enzyme level (0.058 Uml^{-1}) is reported on 3^{rd} days of fermentation (Fig-3.7)





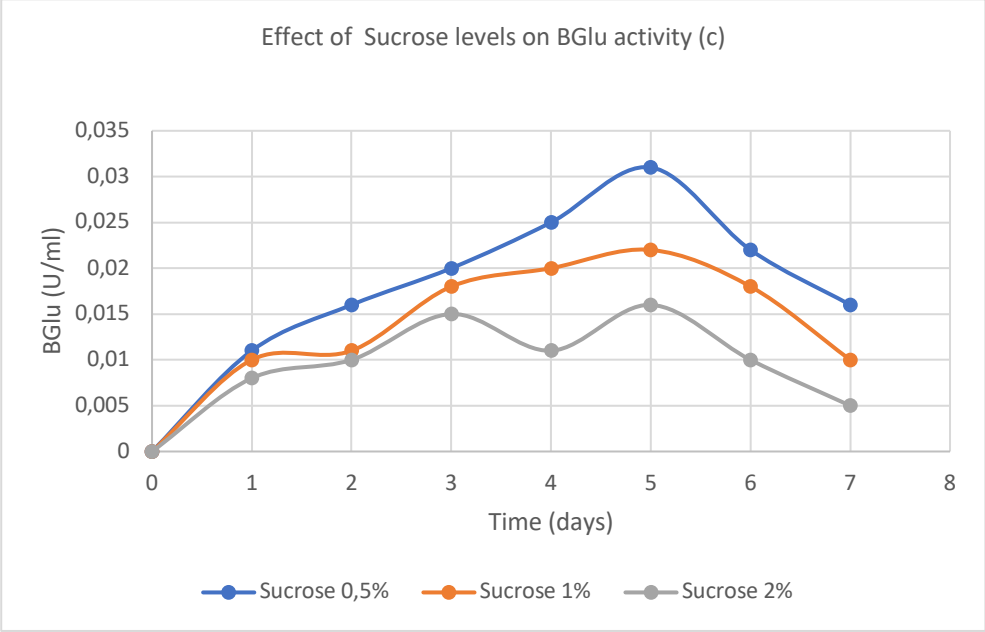
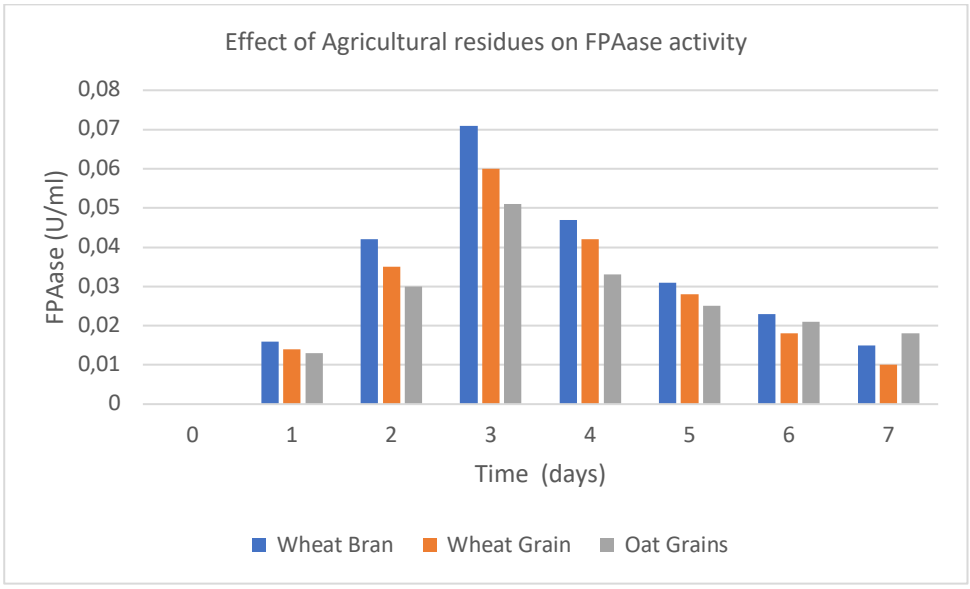
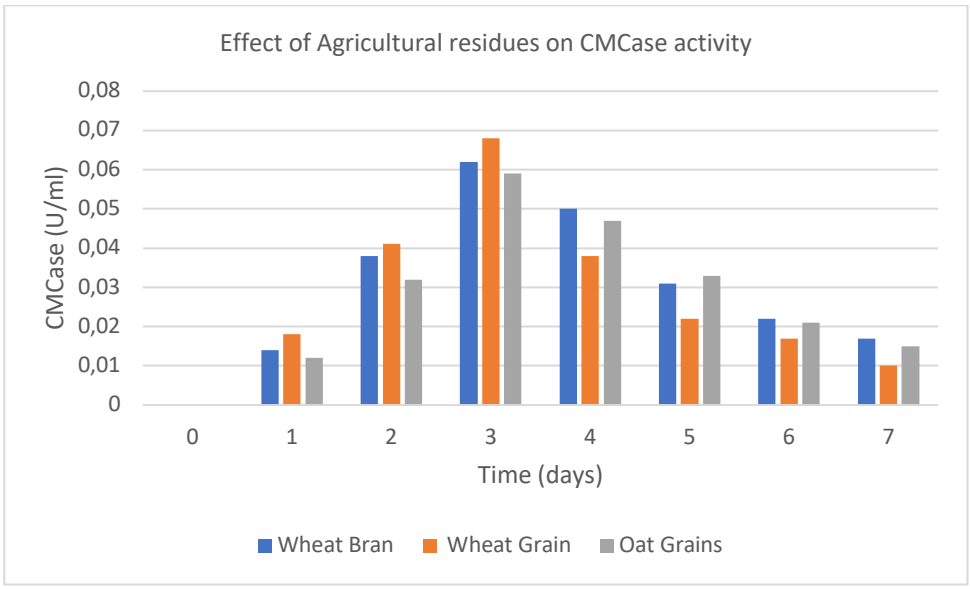


Fig-3.7: Enzyme activity different level of sucrose in SSF process

3.6 Effect and addition of various agricultural residues on fungal enzyme activity in SSF.

The market rejected three different agricultural residues were analyzed to study their effect on fungal growth and activity for enzyme production in optimal environment in flask condition. There was a good enhancement of all three enzymes were found during enzyme estimation. A notable increment for FPase (0.071 Uml^{-1}) and CMCase (0.060 Uml^{-1}) was observed in flask having 1% wheat bran along with fermentation media (Fig-3.8). Higher activity is found on 3rd days of fermentation. Higher β-glucosidase enzyme activity (0.035 Uml^{-1}) is also found on 5th days in same flasks. Wheat bran is proved good enhancer for higher enzyme activity (Fig-3.9).





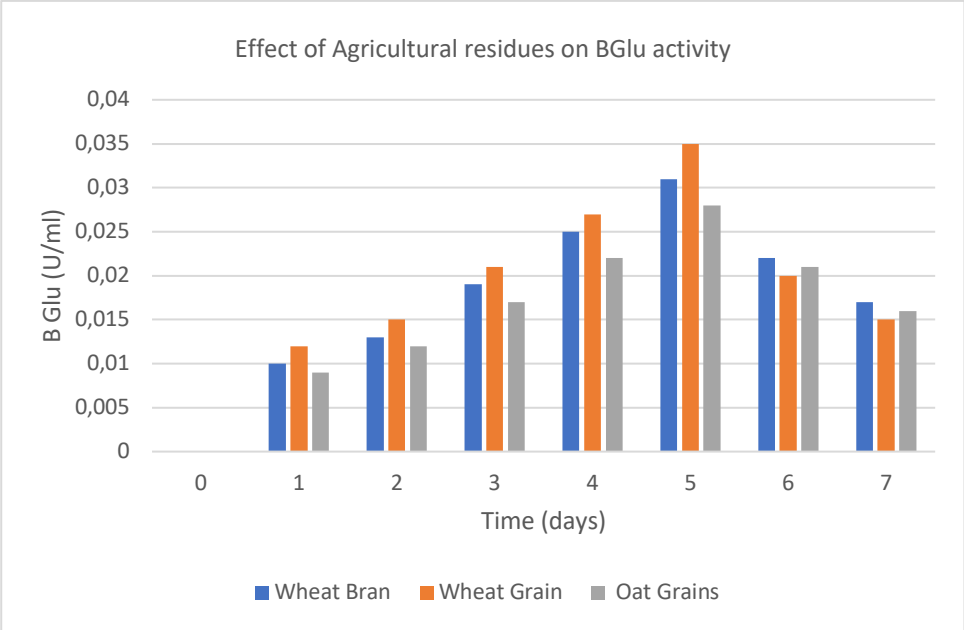
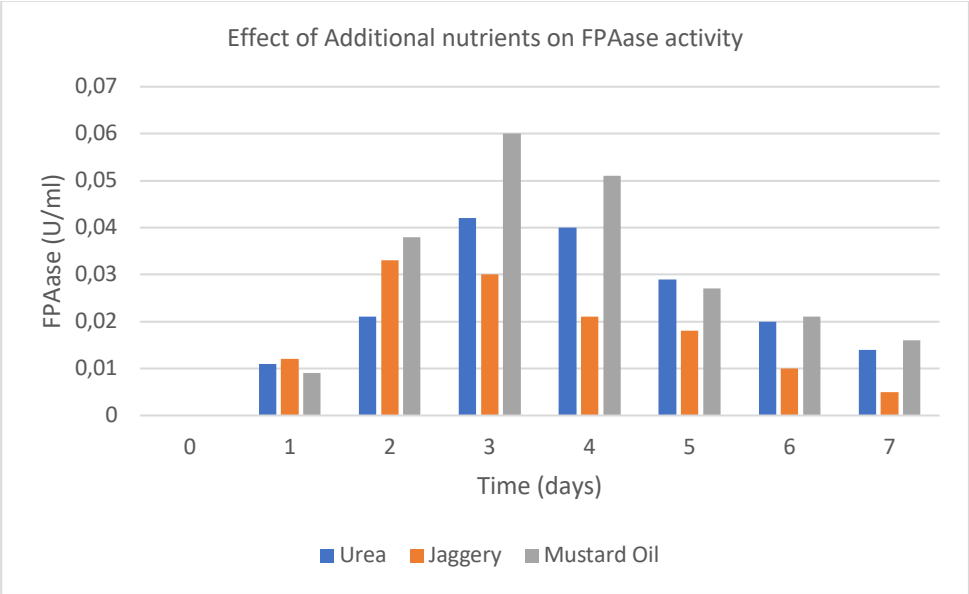


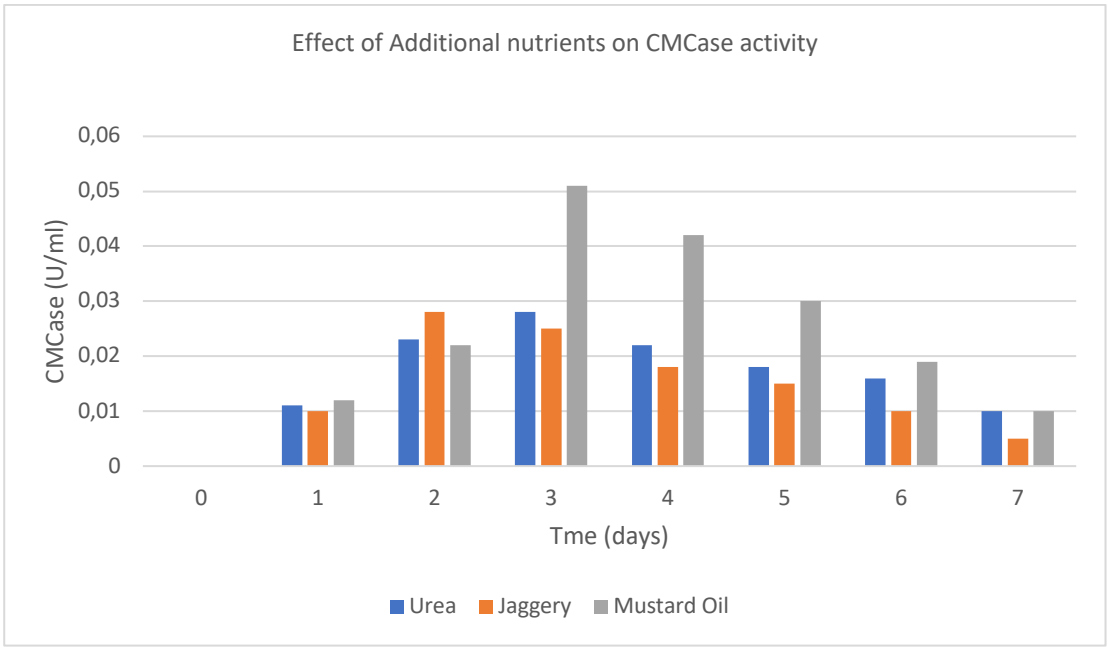
Fig-3.8: Effect on enzyme activity by the addition of agricultural residues in the SSF process

Fig-3.9: Mycelial growth (7th days) *Neurospora sitophila* on media supplemented with wheat bran (1%)

3.7 Effect of the additional nutrients on enzyme activity:

Three different additional nutrients were analyzed for effect on fungal enzyme activity under solid-state fermentation. During this analysis urea, Jaggery and mustard oil (1%) was added in flasks after adjusting the optimal condition. The fermentation was carried out and during enzyme estimation urea and jaggery were observed with inhibitory and their addition clearly hindered enzyme action (Fig-3.10). While on the other hand, Mustard oil (1%) was less inhibitory for enzyme production by fungal strain and higher β-glucosidase activity (0.031 Uml^{-1}) is reported on 5^{th} days of fermentation.





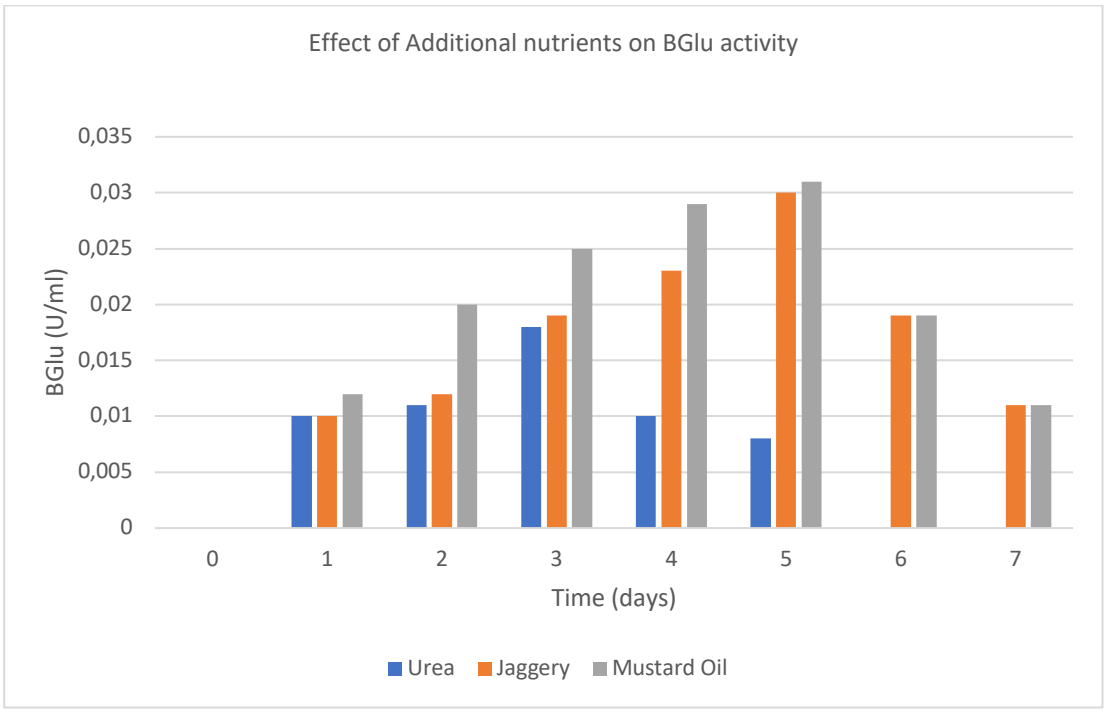
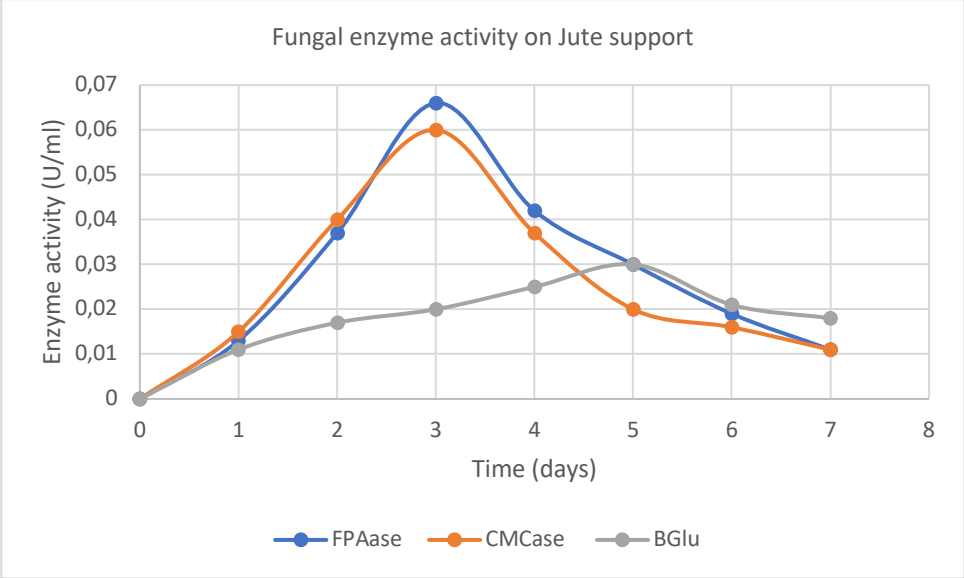


Fig-3.10: Effect of the additional nutrients on enzyme activity

3.8 Analysis of solid support for enzyme activity under SSF condition:

Jute and cotton fabric materials are available abundantly at considerably lower prices were selected. These materials are either burnt or have limited usage. Burning of these materials is of great concern as this is one of the major causes of environmental pollution. Therefore, employment of these materials for solid-state fermentation may essentially designate these from the source of menace to the potential providers and may be a vital source of revenue generation for rural masses. A size of 1cm^2 along was used as the solid matrix for enzyme production (Fig- 3.11). According the result obtained lower activity for all enzymes is reported. This solid support negatively affected enzyme activity might be due to dense and thickness of media.



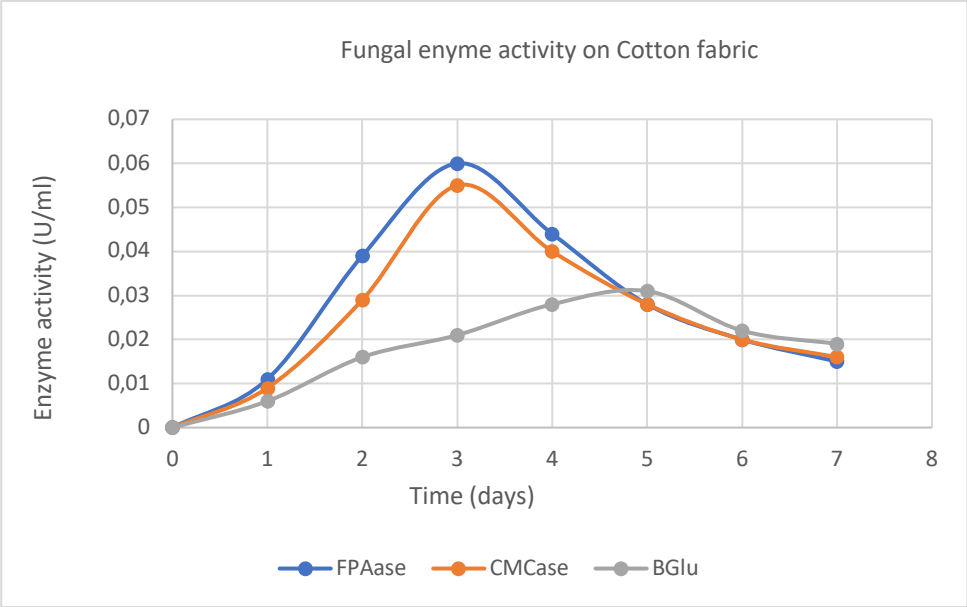


Fig-3.11: Analysis of solid support for enzyme activity under SSF condition

3.9 SSF based Tray bioreactor operation with optimized conditions:

The process of solid-state fermentation was scaled up by designing a tray bioreactor (Fig. 3.13) and the production of gluconic acid was evaluated by using tea waste as solid support during batch fermentation. The various parameters and factors as derived from the earlier studies were utilized for scaled-up process for enzyme production. Fermentation medium along with the inoculum was filled and loaded in two small trays. The humidifier tray contained sterilized water for providing the desired humidity, and sterilized air was circulated through air pump for proper aeration. During the fermentation cycle, the enzyme sample was recovered and estimated every day.

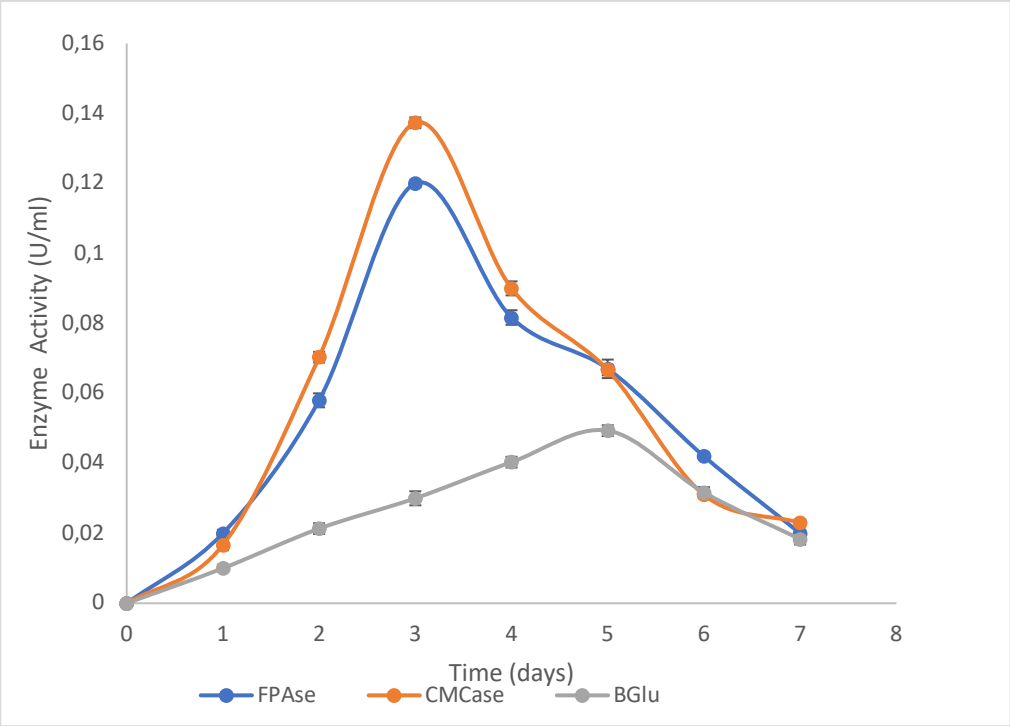


Fig -3.12: Enzyme activity in tray bioreactor operation

Fig -3.13 : Fungal mycelial growth (5th Days) on trays of bioreactor

A significantly higher level of enzyme activity was achieved for all three enzymes. A higher FPase (0.121±.0010 Uml^{-1}) and CMCase (0.139 ±.0015 Uml^{-1}) was achieved during the 3rd days of tray bioreactor operation (Fig-3.12). SSF based tray bioreactor was found much promising with improved and enhanced enzyme activity. Higher β-glucosidase enzyme activity (0.048 ±.0015 Uml^{-1}) was also reported on 5th days of bioreactor operation. Under derived optimized conditions of solid-state fermentation and during fermentation using tray bioreactor had indicated significantly specific and higher enzyme activity.

4. Conclusion :

The second-generation biomass Press cake is evaluated as a substrate for the bioconversion process. In press cake present nutrients and sugars content were targeted to exploit in sustainable way. It was observed that submerged fermentation condition is less productive and unfavorable for fungal *Neurospora* strain . Lower enzyme activity of FPase and CMCase (0.024 Uml^{-1} and 0.018 Uml^{-1}) activity were observed on 3^{rd} day by grown fungal mycelia in submerged state. Among the fermentation media (Water, diluted brown juice and minimal media) evaluated only minimal media with press cake has shown maximal enzyme activity in comparison to others. Unautoclaved flasks were contaminated by other microbes and no enzyme activity was observed. While on the other hand, solid-state fermentation condition is found quite favorable for enhanced enzyme activity. The fungal strain has revealed improved enzyme activity in compare to submerged fermentation. maximal enzyme activity was observed with press cake with minimal media in SSF condition. FPase activity 0.04 Uml^{-1} and CMCase activity 0.06 Uml^{-1} was observed on 3^{rd} day of fermentation. Higher β-glucosidase activity 0.032 Uml^{-1} was reported on 5^{th} days of fermentation when water was used as moisture content. Brown juice diluted with water found promising and shown good enzyme activity in comparison to water. Maximum FPase (0.048 Uml^{-1}) , CMCase (0.051 Uml^{-1}) and higher β-glucosidase activity (0,026 Uml^{-1}) were analysed with brown juice (5%) under SSF condition. Two different temperature level were studied and temperature level of 30^0C is found most optimal for higher enzyme activity and FPase (0.049 Uml^{-1}) and CMCase (0.052 Uml^{-1}) activity is observed during 3^{rd} days in fermentation cycle. While in β-glucosidase activity no major change was observed. A good enhancement in FPAase enzyme activity is observed with 0.5% of Sucrose addition in fermentation media. Higher FPAase enzyme level (0.058 Uml^{-1}) and CMCase (0.06 Uml^{-1}) is observed . A notable increment for FPase (0.071 Uml^{-1}) and CMCase (0.060 Uml^{-1}) was observed in flask having 1% wheat bran along with fermentation media. The other two agricultural waste residues analyzed were not much encouraging to higher enzyme activity in compare to wheat bran. Three different nutrients (Urea, Jaggery and Mustard oil) were also analyzed to study any positive effect on fungal bioconversion process. But there was no clear enhancement was seen by supplementation of these nutrients. however, urea is observed inhibitory for all three enzymes. In similar way the Jute net and cotton fabric used for solid support to fungal organism also shown any

improvement in enzyme activity. The remarkable enhancement in enzyme activity was observed when SSF based tray bioreactor condition was provided to fungal strain. An improved FPase (0.121 Uml^{-1}) and CMCase (0.139 Uml^{-1}) was achieved during the 3^{rd} days of tray bioreactor operation. An improved of (70%) in FPase activity and (131.7%) in CMCase was observed during bioreactor.

5. Perspectivation :

The present research project was planned to utilize press cake and brown juice as a cheaper carbon substrate to further utilize it in value-added product formation. The results obtained from the study has shown that submerged fermentation is energy consuming and less productive for enzyme activity. The press cake and brown juice are available according to seasoned basis. The Alfalfa crop is harvested in (April to Sep) in summertime. But in winters the chances of getting freshly available press cake n brown juice is impossible. The preservation of press cake and brown juice at -18^0C require space and energy. The preserved press cake could be slightly different in chemical nature. Microbial contamination is highly probable in ensilaged material. Unsterilized press cake and brown juice were found totally unsuccessful in order to establish an economical energy-saving process. Contamination threat is always present on PC and brown juice kind of substrate. A higher concentration of brown juice is quite inhibitory in autoclaved condition in media preparation due to caramelization. Wheat bran is observed good inducer for enhanced enzyme activity so Denmark is good agricultural land some other agricultural residues can be evaluated in enzyme production. Solid-state fermentation has been proved highly economical in term of energy consumption. SSF has good advantages like less free water and enviornmental friendly in operation. Tray bioreactor with improved automated system control could be crucial for economic benefits and revenue development. More value-added products and important building blocks can be obtained by utilizing and extracting more sugar from this second-generation biomass. SSF based tray bioreactor with more crucial scalable parameters could give promising enzyme productivity. An improved enzymatic activity was estimated in batch bioreactor could be due to larger surface area for microbial activity in compared to flask condition. It is also evident that improved enzyme activity in compare to flask condition might be due to effective aeration provided to grown mycelial biomass on larger tray surface. Evaluation of aeration effect in bioreactor scaleup studies could be a good initiative. In repeated batch operation sterilization and cleaning of tray bioreactor is big challenge. Frequent contamination was

faced in this tray bioreactor operation. Hence, instead of plastic material metal trays could the best choice. Metal trays can be sterilized and cleaned by different methods to eradicate the contamination problem. *Neurospora sitophila* used in present work is a wild type strain and final enzyme activity under optimized condition is not impressive in comparison to other commercial microbial strains. I strongly believe that a genetically improved mutant strain could be a game-changer for profit and revenue generation. A potent Trichoderma our other good cellulase producer also be effective but the selection of *Neurospora sitophila* was made due to its GRAS category and safer product formation. Since hundreds of years, *Neurospora sitophila* has been used in various edible and food preparation. The main idea was to first to extract produced enzymes from fermented product and secondly utilized to fermented rest slurry in food purpose for animals. Due to limited time period nutritional value assessment was unaccomplished. Instead of using fermented slurry into burning or composting which is quite an uneconomical manner we can utilize fermented part into a nutritional feed for animal farm.

In this study only enzymatic evaluation is done. A planned brief economic statistical-based assessment on productivity, product yield, biomass yield, and other related observation can give brighter feasibility in process optimization.

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