Comparative analysis of cellulase production using *Penicillium funiculosum* wild type & cre A mutant strain in bioreactor

M. Tech Major Project Thesis

Submitted by

Preetam Mallick

Roll. No. 2K15/IBT/10

Department of Biotechnology

Delhi Technological University

Under the Guidance of

Dr. Jai Gopal Sharma

Associate Professor
Department of Biotechnology
Delhi Technological University, Delhi

DECLARATION

I, Preetam Mallick M. Tech IndustrialBiotechnology,Department of Biotechnology, 4th semester, bearing **Roll no-2K15/IBT/10,** Delhi Technological University, Delhi, declare that my project work titled "**Comparative analysis of cellulase production by *Penicllium funiculosum* wild type & cre A mutant in biorector**", is original and no part of this work report has been submitted for any other degree or diploma. All the given information and works are true to my sense and knowledge.

Date:

Place:

CERTIFICATE

This is to certify that the project entitled"**Comparative analysis of cellulase production using *Penicillium funiculosum* wild type & cre A mutant strain in bioreactor"** is the bonafide work of **Mr Preetam Mallick**, a M. Tech II Year (IndustrialBiotechnology,Department of Biotechnology),Roll no -2K15/IBT/10 ,student from Delhi Technological University, Delhi, is a record of his own work carried out under my supervision and guidance during August 2016 to June 2017. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Dr.Jai Gopal Sharma **Prof.D. Kumar**

Associate Professor
Department of Biotechnology
Technological University

Head of the Department
Department of BioTechnologyDelhi
Delhi Technological University

ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and indebtedness to **Prof. D. Kumar**(Head of the Department, Department of Biotechnology,DTU,Delhi), **Dr. Jai Gopal Sharma**(Associate Professor, Department of Biotechnology, DTU, Delhi) & **Dr.Syed shams Yazdani**(Group leader Microbial engineering integrative biology group,coordinator-DBT – ICGEB Centre for advance bioenergy research,ICGEB,Delhi) introducing the present topic and for his inspiring guidance, constructive and valuable suggestion throughout this work.

I am heartily thankful to **Mr.Danish Eqbal & Mr. Rajkumar Bildan** for their guidance and support during my project. His able knowledge and expert supervision with unswerving patience fathered my work at every stage, for without his warm affection and encouragement, the fulfilment of the task would have been very difficult.

I owe my sincere gratitude & thankfulness to **Amar Pratap Singh** for giving me the opportunity to work with him and for always being there whenever I approached him in any dilemma.

I am genuinely appreciative of all my Friends for their suggestions and moral support during my work.

Contents Page number

3.Review of literature	11-25
3.1.Generation of biofuels	11-12
3.2.Lignocellulosic feed stock	12-13
3.3.Steps in 2nd generation bioethanol production	14-17
3.4.Media Optimisation	17-20
3.5.Review on media optimisation for *Penicillium funiculosum*	20-21
3.6.Different reactor operatio	21-22
3.7.Volumetric effect	23
3.8.Penicillium funiculosum review	23-24
3.9.Review on cre A mutation	25
4.Materials & Method	
4.1.SOP OF FPU Assay	27-29
4.2.SOP OF CMCS Assay	29-31
4.3.SOP OF Xylanase Assay	31-33
4.4.Sporulation SOP	34
4.5.Volumetric effect & media optimisation	34-36
4.6.Packed Cell Volume &Dry weight	36-38
4.7.Bioreactor with wildtype of *P funiculosum*	38-41
4.8.Bioreactor with del cre A of *P funiculosum*	41-42
5.Result & Discussion	44-53
6.Conclusion	55
7.Future perspective	57
8.Reference	59-60

Abstract:

Biofuel production has been given importance & also increase globally from last decade. First generation biofuel that is mainly produced from the food crop of simple sugar & vegetable oil reported to effect on food security & environment. Now a days the main oil production companies are mainly focused on second generation biofuel production which is produced from mainly lignocellulosic feedstock, that reduce carbon dioxide production,improve economy & increase the use of food crope by product & agricultural waste. Now Indian Ministry of Petroleum & natural gas has fixed a limit that 10% of ethanol should be blend with petrol. Oil & natural gas industries achieve only 2.3% of this fixed value of this predetermined limit during 2014-15.This second generation bioethanol mainly produced from lignocellulosic food crops by an universal microorganism *Saccharomyces cerevisiae*, which can only convert simple sugar such as glucose to ethanol though glycolysis & dark reaction.So,lignocellulosic feed stock that's composed of cellulose,hemicellulose & lignin first pretreated to remove lignin & then cellulose & hemicellulose then converted to glucose by an enzyme complex known as cellulase. Our work was focused on enhancement the production of cellulase from filamentous fungi *Penicilliun funiculosum* wild type & cre A mutant strain.To achieve this we mainly optimized & selected the optimum media by providing one rich media RCM & one minimal madia MM medium & also analysed the volumetric effect in small scale by providing 1:5 media:surface volume .We also determined the optimum Packed Cell Volume(PCV) & dry weight in three different spore concentration such as 10^5,10^6 & 10^7.Afer media selection & optimization we proceed to scale up the production of cellulase from *Penicillium funiculosum* wild type & cre A mutant strain in 21 litre fermentor for industrial production & also to determine optimum day of cellulase production.It has been seen from experiment that RCM media has given high CMC(IU/ml),FPU & Xylanase(IU/ml) activity than MM media & the CMC(IU/ml),FPU &Xylanase(IU/ml) were also greater in RCM & MM 50 ml volumes than RCM & MM 200 ml volumes for analysing 1:5,volume to surface ratio. For PCV & dry weight optimization it has been shown 10^7 spore/ml concentration of *Penicillium funiculosum* wildtype strain given optimum PCV & dry weight after 48 hours inoculation than 24 hours. In case of 21 litre fermentor run with *Penicillium funiculosum* wild type in Modified RCM media of 1:10 volume:surface ration given less CMC(IU/ml) & FPU and shown optimum PCV, CMC(IU/ml) & FPU ON 9th day.For 21 litre fermentor operation with cre A mutant strain of *Penicillium funiculosum* in same condition as of wild type,shown enhanced production of CMC(IU/ml),FPU than wild type.And CMC(IU/ml) & FPU were optimum on 6th day & 5th day respectedly with cre A mutant strain.

Part-2

Introduction

Introduction- Recently due to limitation of simple sugars from sugar crop(sugarcane) and starch (corn,wheat) the first generation biofuel is replaced by second generation biofuel such

as bioethanol,which is produced from lignocellulosic feedstock such as agricultural residues(sorghum,barly,ricestraw,oilseed ,sugar crop etc),agricultural processing by product(rich hull),dedicated energy crop(switchgrass,poplar,willow & forest products and residues. Lignocellulose is the main component of plant cell wall, consist three major components :

a)Cellulose-Lignocellulose contain about 40-60% of cellulose of total dry weight.It is a polysaccharide containing long chain of beta 1-4 linked D-glucose monomers gathered inside of the micrifibrils bundles. Cellulose molecules lead to a strong matrix structure & requires 320◦C as well as high pressures (upto 25MPa) to transform into an amorphous structure in water .

b) Hemicellulose- Lignocellulose contain about 20-40% of hemicellulose of total dry weight. It is an amorphous structure formed of different heteropolymers such as hexoses (D-glucose, D-galactose, and D-mannose) as well as pentose (D-xylose and L-arabinose). It may contain sugar acids (uronic acids).Hemicellulose backbone chain is primarily composed of xylan which is a polymer containing 90% of α-xylose & 10% of L-arabinose.

c) Lignin-It is an aromatic and rigid biopolymer, covalently bonded to hemicellulosic xylans .It is a biopolymer containing phenyl propionic alcohol such as coniferyl, coumaryl & sinapyl alcohol.Lignin provide rigidity and high level of compactness of plant cell wall & hence serve as a plant major defence system to pathogen & insects.

The production of bioethanol from lignocellulosic feedstock consist several steps:1)milling,2)pretreatment,3)enzyme hydrolysis,followed by 4)fermentation.As universal organism for bio ethanol production ,yeast *Saccharomyces cerevisiae* & bacteria *Zymomonas mobilis* can only metabolize the simple sugar like glucose,xylose & arabinose.So cellulose & hemicellulose first converted to fermentable sugar such as glucose,xylose & arabinose.For this purpose lignocellulosic biomass primarily pretreated by different procedure such as acid hydrolusis(with concentrated sulphuric acid or diluted sulphuric acid),ammonia treatment,lime treatment,alkaline peroxide treatment,wet oxidation & stream explosion process.Through this pretreatment process mainly lignin is removed as lignin form phenolic group after degradation,which hamper the enzymetic activity of cellulase enzyme complex.As the lignin is removed by pretreatment the cellulose & hemicellulose undergoes enzymetic hydrolysis to produce simple sugar monomer glucose,xylose by the enzyme complex called **cellulase.**

Cellulase is enzyme complex,mainly excreted my fungi & some bactia,consists mainly

a) **Endoglucanase, or CMCase-**Endo-(β-1,4)-gluconase breaks β-1,4-bonds of cellulose chain thus producing new end.It contain cleft/grove-shaped active site.

b) Exo-(β-1,4)gluconase or cellobiohydrolase-It act on the reducing or non reducing end of cellulose polymerchain generating either cellobiose or glucose.the active site of enzyme contain a loop like structure.

c) **β-Glucosidase-**This is required to hydrolyze cellobiose to glucose. β-Glucosidase have a pocket-shaped active site,that bind to the nonreducing glucose unit and clip glucose off from cellobiose or cellodextrin.

d) **Xylanase**-Xylanase are mainly hemicellulase which catalyse the endoendohydrolysis of 1,4-β-D-xylosidic linkages in xylan. Xylan is a linear polymer of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds, is a type of hemicellulose.

This cellulase is produced by fungi,bacteria,plant,protozoans, may be in free or cell surface bounded form.Among the microorganisms filamentus fungus ***Penicillium funiculosum*** shown a high potentiality in cellulase production.

Cellulase production from ***Penicillium funiculosum*** can be enhanced by by several ways such as 1) media optimization,2) strain improvement by Carbon Catabolism Represser cre A deletion.

For the industrial production of cellulase from Penicillium funiculosum we mainly scale up it in stirred-tank rector with batch operation process.

Stirred-tank reactor – The traditional fermenter is the stirred-tank reactor ,which is an example of a bioreactor with internal mechanical agitation.This system provide high flexibility and high k(L)A(volumetric mass transfer coefficient) values for gas and stirred tank reactor can used upto 2000 centipoises(2 Pa sec) viscosity.

The vessel is made of stainless steel.Type 316 is used on all wetted part & type 304 is used on covers and jacket. Glass fermenters are rarely used at the 50l .Most of the fermenters are built with a height-to-diameter ratio of 2 to 3.The working volume in a fermenter is typically about 75% of total volume of fermenter.

This traditional stirred –tank reactor has following component:

A)Sparger-The pressurized gas is supplied through sparger.A sparging ring will provide smaller bubble size & better gas distribution. Sparger with single discharge point is preffered for media with high levels of suspended solids.

B)Impeller-A impeller mainly provide rapid agitation to disperse the bubbles throughout the tank,to increase their residence time within the liquid,and to shear larger bubbles in to small bubles.There are two main type impeller

1)Rushton impeller- Rushton impeller is a disc typically consist 6 to 8 blades to pump the fluid in a radial direction.The impeller diameter is typically 30% to 40% of the tank diameter

2)Axial flow hydrofoil impellers: This system cam pump the liquid either down or up and have shown to give superior performance with respect to lower energy demands for the level of oxygen transfer.It break up to compartmentalization often observed with multiple radical flow impeller system are some time used.It consist four baffles which are 8% to 10% of reactor diameter.

C) Cooling jacket & coil-Large reactors use either internal coil for heat removal or a jacket vessel.Although copper coil have better thermal transport characteristics,strainless steel coils are also used,but its depende on the nature of media and culture.

Part-3

Review of literature

1.Biofuel&generation of biofuel

1.1.Biofuel:A **biofuel** is defined as any fuel or hydrocarbon that is made BY or FROM a living organism that we humans can use to power something.

Biological Carbon Fixation

Carbon fixation is a process that takes inorganic carbon (in the form of things like CO_2) and converts it into organic compounds. In other words, any process that converts carbon dioxide into a molecule that would be found in a living organism is carbon fixation. If this process occurs in a living organism, it is referred to as 'biological carbon fixation'.

Fuel

The next part of the definition of a biofuel involves fuel. A fuel is nothing more than something from which we humans can get energy. Carbon fixation can lead to a number of different compounds, like proteins, fats, and alcohols (just to name a few). If any of those molecules can be used to provide energy in a mechanical setting, we call it a fuel.

1.2. Generation of biofuel:

There are three types of biofuels: 1st, 2nd and 3rd generation biofuels. They are characterized by their sources of biomass, their limitations as a renewable source of energy, and their technological progress. The main drawback of 1st generation biofuels is that they come from biomass that is also a food source. This presents a problem when there is not enough food to feed everyone. 2nd generation biofuels come from non-food biomass, but still compete with food production for land use. Finally, 3rd generation biofuels present the best possibility for alternative fuel because they don't compete with food. However, there are still some challenges in making them economically feasible.

1.2.1. First generation biofuel-First generation biofuels are produced directly from food crops. The biofuel is ultimately derived from the starch, sugar, animal fats, and vegetable oil that these crops provide.First generation biofuels are produced through well-understood technologies and processes, like fermentation, distillation and transesterification.. Corn, wheat, and sugar cane are the most commonly used first generation biofuel feed stock.

1.2.1.1.Disadvantages of first generation biofuel:
a) They pose a threat to food prices since the biomass used are food crops such as corn and sugar beet. First generation biofuel production has contributed to recent increases in world prices for food and animal feeds .
b)They also have the potential to have a negative impact on biodiversity and competition for water in some regions.
c) They also only provide a small benefit over fossil fuels in regards to greenhouse gases since they still require high amounts of energy to grow, collect, and process.

1.2.2. Second generation biofuel- Second generation biofuels are also known as advanced biofuels. What separates them from first generation biofuels the fact that feedstock used inproducing second generation biofuels are generally not food crops mainly lignocellulosic feedstock.

1.2.3. Third generation biofuel:

Third generation biofuels use specially engineered crops such as algae as the energy source. These algae are grown and harvested to extract oil within them . The term **third generation biofuel** has only recently enter the mainstream it refers to biofuel derived from algae. However, when it became apparent that algae are capable of much higher yields with lower resource inputs than other feedstock. The diversity of fuel that algae can produce results from two characteristics of the microorganism. First, algae produce an oil that can easily be refined into diesel or even certain components of gasoline. More importantly, however, is a second property in it can be genetically manipulated to produce biofuel.The list of fuels that can be derived from algae includes,biodiesel,butanol,gasoline,methane,ethanol,vegetableoil,jet fuel.

1.2.3.1.Cultivation of Third Generation Biofuels

Another favorable property of algae is the diversity of ways in which it can be cultivated. Algae can be grown in any of the following ways.

a)**Open ponds** – These are the simplest systems in which algae is grown in a pond in the open air. They are simple and have low capital costs, but are less efficient than other systems. They are also of concern because other organisms can contaminate the pond and potentially damage or kill the algae

b)Closed-loop systems – These are similar to open ponds, but they are not exposed to the atmosphere and use a sterile source of carbon dioxide. Such systems have potential because they may be able to be directly connected to carbon dioxide sources (such as smokestacks) and thus use the gas before it is every released into the atmosphere.

c)Photobioreactors – These are the most advanced and thus most difficult systems to implement, resulting in high capital costs. Their advantages in terms of yield and control, however, are unparalleled. They are closed systems.

2.Different types of lignocellulosic feedstock : The three main components of lignocellulosic biomass are cellulose,hemicellulose, and lignin. The three main sources of lignocellulosic biomass are **forest products and residues, agriculturalresidues, and dedicated energy crops.** The availability of lignocellulosic feedstocks for biofuel production in the United States and otherregions of the world are discussed in this section.

2.1. Agricultural Residues-Small grain residues include sorghum, barley,oat, and rice straw. Other crop residues include cotton, other oil seeds,tobacco, sugar crops, potatoes, beans, miscellaneous root crops, and double crops. Corn stovertypicallycontains 36 weight percentglucan, 19 weight percentxylan, 2.3 weightercentarabinan, 0.9 weight percentgalactan, and 0.5 weight percentmannan;thus the theoretical ethanol yield will be 112 gal (426 L)/dryMT of corn stover. Under the most favorable conditions it is estimated that by the midtwenty-first

century, 345million dry MT/yr agricultural biomassresidues will be available in the United

States. Corn stover still
dominates with 232 million dry MT/yr.
Table-1

Chemical Composition of Agricultural Biomass Residues is given below in the table:

Residue	**Cellulose (%)**	**Hemicellulose (%)**	**Theoretical ethanol yiel(gal/MT dry)d***
Barley straw	38.9	20.9	104
Corn stover	36.9	21.3	101
Oat straw	38.2	30.0	119
Rice straw	38.6	19.7	101
Sorghum straw	34.0	20.0	94
Wheat straw	39.6	26.4	115
Bagasse	39.1	22.5	107

2.2.Agricultural Processing By-Products: In postharvest processing of crops large quantities of by-products aregenerated. The carbohydrates contained in these by-products can be converted to ethanol. Corn fiber is a by-product of the wet-milling process whereas DDGS is a by-product of the dry-grind process. However, with the current rate of increase in ethanol production in the United States, the feed market may soon be saturated and conversion of the excess corn fiber and DDGS toadditional ethanol will be of great interest.Rice hull has great potential as a feedstock for ethanol production because it is available in very large quantities. Typically rice hull accounts for about 20 percent of thegrain weight.2 In 2005 rice production in the world was 153 millionMT, of which Asia contributed 137 million MT. The rice hulls available in Asia have a theoretical ethanol production of 49.7 billion liters(13.1 billion gallons).

2.3. Dedicated Energy Crops: In addition to forest and agricultural biomass residues, dedicated
energy crops are another lignocellulosic biomass source for biofuel production. Among a rather large number of herbaceous and woody crops that can be used for biofuel production,switchgrass (*Panicumvirgatum*), poplar (*Populus*spp.), and willow (*Salix* spp.) have beenthe focus of most research efforts. Among the three potential energy crops, switchgrass has the lowest production cost. Poplar and willow have higher production cost because of the higher establishment costs and the longer period of time before harvest is possible.35 Switchgrass has great potential for
biofuel production. Alamo switchgrass, which is most suited for the Southeastern part of the United States, can be produced at 5.4 to 7.2 dry MT per acre.
Table-2

2.3.1.Following table is for Carbohydrate Composition of Dedicated Energy Crops and Their Theoretical Ethanol Yields

Energy crops	**Cellulose (%)**	**Hemicellulose (%)**	**Theoretical ethanol yield* (gal/MT dry)**
Hybrid poplar	39.8	18.4	101
Willow	43.0	21.3	112
Switchgrass	32.2	24.4	99

3.Steps in Ethanol Production from LignocellulosicFeedstocks& function of cellulases in ethanol production

To convert lignocellulosic feedstock to ethanol there are few steps :1)**Biomass Pretreatment, 2) Enzyme Hydrolysis, 3)Fermentation.**

3.1. Biomass Pretreatment: The main objectives of the pretreatment process are to speed up the rates of hydrolysis and increase the yields of fermentable sugars. In all pretreatment processes, these goals are accomplished by modifyingthe structure of the polymer matrix in the biomass, thus making thecarbohydrate fractions more susceptible to acid attack or more accessible to enzyme action. Lignin normally is considered a wasteand is burned to supply thermal energy.

a.1)**Concentrated Sulfuric Acid Hydrolysis-** In this process, decrystallization of cellulose and hemicellulose is carried out by adding 70 to 77 percent sulfuric acid to a biomass that has been dried to 10 percent moisture.

a.2) Dilute Sulfuric Acid Hydrolysis- To reduce enzyme requirements, a two-stage process was devee washed solids are fed to the second-stage acid impregnation at 40 to 60 percent solids by weight. The solids are treated under more severe conditions (190 to 240°C) loped at the National Renewable Energy Laboratory (NREL) in Golden, Colorado. The biomass solid content in the first-stage impregnation is 30 to 35 percent by weight. After drying, the biomass arrives at the firststage hydrolysis at 40 to 60 percent solids by weight. The biomass is treated at 130 to 220°C in the first-stage hydrolysis. It is then discharged into a flash tank at 120 to 140°C. The solids then are discharged into a flash tank at 120 to 140°C.

b) Steam Explosion-In the steam-explosion pretreatment process, biomass is exposed to superheated steam in a reactor. The highpressure steam penetrates the biomass and initiates an autohydrolysis reaction. It is believed that improvement of enzyme hydrolysis is caused by removal of most of the hemicellulose and some of the lignin rather than disruption of the biopolymer structure by the explosion.Good results can be obtained at either high temperature and short reaction time (270°C, 1 min) or low temperature and long reaction time (190°C in 10 mins).

c) Ammonia Treatment- Bases such as sodium hydroxide, potassium hydroxide, and ammonia can be used for biomass pretreatment. Base solutions cause swelling of biomass, which subsequently leads to decrease in the degree of polymerization, decrease in crystallinity, disruption of the lignin structure, and separation of structural linkages between lignin and carbohydrates. A flow-through process called Ammonia Recycle Percolation (ARP) was developed for pretreatment of corn stover.84 In this process, 15 percent (by weight) ammonia is pumped through a bed of biomass maintained at 170°C and 2.3 Mpa. The ammonia fiber/freeze explosion/expansion (AFEX) process uses anhydrous ammonia instead of aqueous ammonia.88 The concept of AFEX is similar to steam explosion where biomass is come to contant with anhydrous ammonia at 60°C to 120°C.

d)Li me Treatment -Biomass can also be pretreated with lime to improve subsequent enzyme hydrolysis to fermentable sugars. Typical lime loading is 0.1 g Ca(OH)2 per gram biomass. For example, corn stover treated with excess lime at 0.5 g Ca(OH)2 per gram biomass at 55°C with aeration needed 4 weeks.92 At the end of the treatment, 87.5 percent of the lignin was removed and some of the carbohydrate fractions were also solubilized.

e) Alkaline Peroxide Treatment-The use of alkaline solutions of hydrogen peroxide was one of the early attempts to develop pretreatment processes for biomass. In this treatment, large fractions of the hemicellulose and lignin are solubilized whereas most of the cellulose remains intact. The cellulose in the residual solid can be hydrolyzed with enzymes at very high rates and near theoretical yields. The optimum pH is 11.5, which is the pKa for the dissociation of H2O2. When this pretreatment was applied to corn stover, most of the hemicellulose and as much as 50 percent of the lignin were solubilized. The residual solid fraction, which still contains most of the original cellulose, was hydrolyzed with cellulase to above 90 percent of theoretical glucose yield.

f)Wet Oxidation -In the wet oxidation process, biomass is treated with water and air or oxygen at elevated temperatures and pressures.95 Similar to the alkaline peroxide treatment process, large fractions of hemicellulose and lignin are solubilized during wet oxidation, leaving a solid residue high in cellulose. The cellulose in the residual solid can be hydrolyzed with enzymes at high rates and yield.

3.2.Enzyme Hydrolysis Cellulose can be hydrolyzed to glucose by the enzyme complex called cellulase, which is excreted by organisms capable of degrading cellulose. This enzyme complex consists of an endo-b-(1→4)-glucanase (Cx-cellulase) and an exo-b-(1→4)-glucanase (cellobiohydrolase). The Cx-cellulase breaks the bonds in the amorphous regions of the cellulose molecules, and the cellobiohydrolase removes cellobiose from the nonreducing ends. Another enzyme, called cellobiase [b-(1→4)-glucosidase] is required to hydrolyze cellobiose to glucose.
Complete breakdown of this heterogeneous biopolymer of hemicelluloses requires the action of several hydrolytic enzymes called hemicellulases and consist of endo-enzymes which cleave internal glycosidic bonds, exo-enzymes, which remove sugar residues from the nonreducing ends, and esterases, which attack nonglycosidic ester linkages. The hemicellulose hydrolytic enzymes include endob-1,4-xylanase, exo-b-D-xylosidase, a-L-arabinofuranosidase, endo1,5-a-L-arabinanase, a-glucuronidase, acetyl esterases, which consist of acetylxylan esterase and acetyl esterase, and phenolic acid esterases, which consist of feruloyl esterase and p-coumaroyl esterase.
Commercial cellulases normally also contain hemicellulase activities. In fact, the hemicellulose in pretreated corn stover has been routinely hydrolyzed to 70 to 80 percent theoretical yield with commercial cellulases.

a) Endoglucanase-Endoglucanase, or CMCase, randomly cut β-1,4-bonds of cellulose chains, generating new ends. Different endoglucanases are produced by archaea, bacteria, fungi, plants, and animals with different catalytic modules belonging to families 5–9, 12, 44, 45, 48, 51, and 74. Fungal endoglucanases in general possess a catalytic module with or without a CBM, while bacterial endo-glucanases may possess multiple catalytic modules, CBMs, and other modules with unknown function.

The catalytic modules of most endoglucanases have a cleft/grove-shaped active site, which allows the endoglucanases to bind and cleave the cellulose chain to generate glucose, soluble cellodextrins or insol-uble cellulose fragment. However, some endoglucanases can act “processively,” based on their ability to hydro-lyze crystalline cellulose and generate the major

products as cellobiose or longer cellodextrins (Cohen et al., 2005; Li and Wilson, 2008; Mejia-Castillo et al., 2008; Parsiegla et al., 2008; Yoon et al., 2008; Zverlov et al., 2005).

b)Exoglucanase- Exoglucanases act in a processive manner on the reduc-ing or nonreducing ends of cellulose polysaccharide chains, liberating either cellobiose or glucose as major products. Exoglucanases can effectively work on micro-crystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure. CBH is the most-studied exoglucanase. Different CBHs are produced by many bacteria and fungi, with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases. Aerobic fungal CBHs are in families 6 and 7 only; aerobic bacterial CBHs are in families 6 and 48; anaerobic fungal CBHs are in family 48; and anaerobic bacterial CBHs are in family 9 as well as.

The most significant topological feature of CBHs' catalytic module is the tunnel structure which is formed by two surface loops.Fungi are considered good producers of industrially valuable enzymes with higher enzymatic activities. *Aspergillus fumigatus* produced exoglucanase having high enzymatic activity (83 U/gds) during the solid-state fermentation of wheat straw under optimum physical and nutritional conditions. Maximum production was obtained after 72 h of fermentation, at 55 °C temperature, pH 5.5, 80 % moisture level, and 2 mL fungal inoculums. The *T. reesei* genome encodes 10 cellulases and 16 hemi-cellulases (Martinez et al., 2008). It produces two exoglucanases(CBH I and CBH II).

c) **β-Glucosidase**-β-Glucosidases (BGs) that do not contain a CBM hydrolyze soluble cellodextrins and cellobiose to glucose. The activity of BG on insoluble cellulose is negligible. BGs degrade cellobiose, which is a known inhibitor of CBH and endoglucanase.

BGs have a pocket-shaped active site, which allows them to bind the nonreducing glucose unit and clip glucose off from cellobiose or cellodextrin. β-Glucosidases have been classified into glycoside hydrolase (GH) families GH1, GH3, GH5, GH9, and GH30, based on their amino acid sequences, while other β-glucosidases remain to be classified. The GH1, GH5, and GH30 β-glucosidases fall in GH Clan A, which consists of proteins with $(\beta/\alpha)_8$-barrel structures. In contrast, the active site of GH3 enzymes comprises two domains, while GH9 enzymes have $(\alpha/\alpha)_6$ barrel structures. . Different BGs are produced by various archaea, bacteria, fungi, plants, and animals, with different catalytic modules belonging to families 1, 3, and 9.

d) Xylanase- Xylanase is an enzymatic complex is responsible for the hydrolysis of xylan, but the main enzymes involved are endo-1,4-β-xylanase and β-xylosidase. These enzymes are produced by fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, insect, seeds, etc., but the principal commercial source is filamentous fungi.

Xylan is the principal type of hemicellulose. It is a linear polymer of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds. In nature, the polysaccharide backbone may be added to 4-*O*-methyl-α-D-glucuronopyranosyl units, acetyl groups, α-L-arabinofuranosyl, etc., in variable proportions. Xylan is found in large quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual

plants (<30%). It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, in particular in monocots. Wood xylan exists as *O*-acetyl-4-*O*-methylglucuronoxylan in hardwoods and as arabino-4-*O*-methylglucuronoxylan in softwoods, while xylans in grasses and annual plants are typically arabinoxylans.

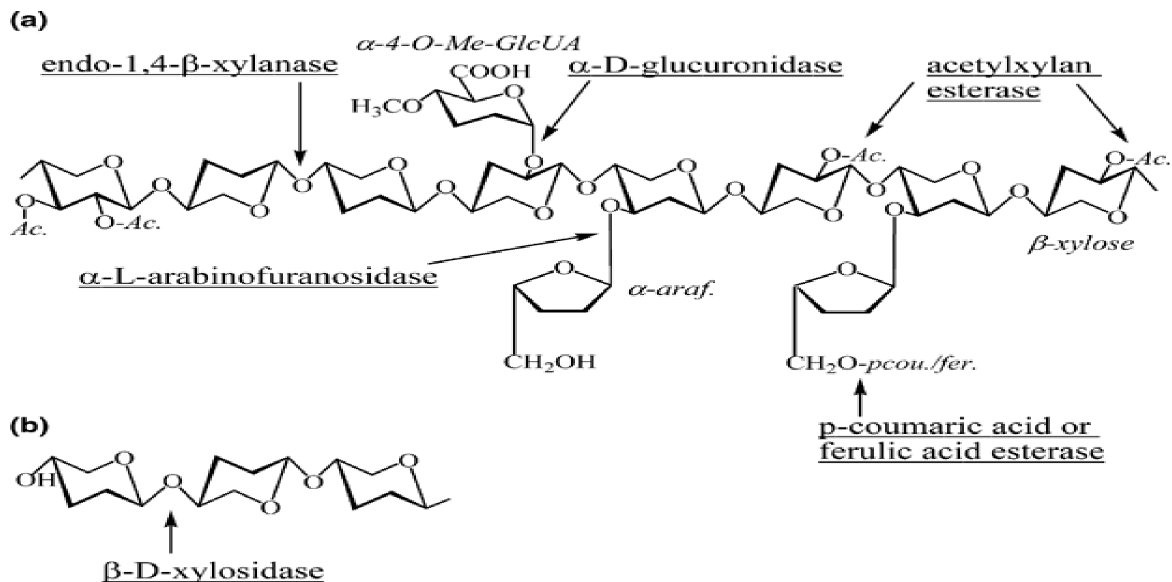


Fig-1 Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4- β-linked xylose residues. Ac., Acetyl group; α-araf., α-arabinofuranose; α-4-*O*-Me-GlcUA, α-4-*O*-methylglucuronic acid; pcou., *p*-coumaric acid; fer., ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase. Adapted from [18]. Figures were prepared with CS Chemdraw Ultra version 6.0.

Typical examples of micro-organisms which produce xylanase isoenzymes include *Aspergillus niger*, which produces fifteen extracellular xylanases , and *Trichoderma viride*, which secretes thirteen. Moreover, as well as multiple catalytic domains, many xylanases are also characterized by the presence of various supplementary domains. Examples include xylan binding domains, cellulose binding domains , dockerin domains (implicated in binding to multidomain complexes produced by certain micro-organism, e.g., *Clostridium thermocellum*) , thermostabilising domains.

4.Optimization of Fermentation Medium

Optimization of production medium is required to maximize the metabolite yield. Fermentation technology is widely used for the production of various economically important compounds which have applications in the energy production, pharmaceutical, chemical and food industry. Although, fermentation processes are used from generations, the need for sustainable production of products, meet the market requirements in a cost effective manner has put forward a challenging demand.

4.1.Nutritional control of metabolite production:

4.1.1. Carbon source- Carbon is the most important medium component, as it is an energy source for the microorganisms and plays an important role in the growth as well as in the production of primary and secondary metabolite. A classic example for this is, penicillin production, where glucose is found to have repression effect. Later, it was found that lactose is a slowly assimilating carbon source and helped in the production of secondary metabolites (i.e., penicillin). Hence, in order to overcome the carbon catabolite repression phenomenon, the production process was established using lactose fermentation.

4.1.2.Nitrogen source-Like carbon, the selection of nitrogen source and its concentration in the media also play a crucial role in metabolite production. The microorganism can utilize both inorganic and/or organic sources of nitrogen. Singh et al. during the optimization of actinomycin V production by *Streptomyces triostinicus* found that biosynthesis of actinomycin V involves tryptophan pathway and addition of amino acid tryptophan to the medium enhances the production. On the contrary, the same amino acid showed inhibitory effect in the production of candicidin from *Streptomyces griseus* (Sanchez and Demain).

4.1.3. Phosphate- Phosphate is another basic component which is required for the production of phospholipids present in the microbial cell membranes, and for the production of nucleic acids. Sanchez and Demain reported that various secondary metabolites' production such as, actinorhodin, cephalosporin, clavulanic acid, streptomycin, tetracycline, vancomycin etc. is highly influenced by inorganic phosphate concentration present in the production medium. In most cases, lower concentration of phosphate is required for the initiation of the metabolite (antibiotic) production and beyond a certain concentration it suppresses the secondary metabolism and ultimately inhibits the production of primary or secondary metabolite.

4.2.Media optimization strategies: Media optimization can be achieved by using a wide range of techniques from classical "one-factor-at-a-time"viz removal,supplement & replacement to modern statistical and mathematical techniques, viz. artificial neural network (ANN), genetic algorithm (GA) etc.

4.2.1. One-factor-at-a-time (OFAT) -

In the classical medium optimization technique, one-factor-at-a-time (OFAT) experiments, only one factor or variable is varied at a time while keeping other variables constant. The concentrations of the selected medium components were then changed over a desired range. Because of its ease and convenience, the OFAT has been the most preferred choice among the researchers for designing the medium composition and used in the initial stages in diverse fields.

a) **Removal experiments**-In this type of experiment, all the medium components are removed from the production medium one-by-one, and after proper incubation period, their effects on the production of secondary metabolite or the product of interest is observed in terms of suitable parameters.For example reported that during the production of antifungal compound from *Streptomyces capoamus*, removal of soybean meal or glycerol or NaCl from the fermentation medium decreased the yield by 20–40%

(Singh et al.).

b) **Supplementation experiments**-Thse are generally performed to evaluate the effects of various carbon and nitrogen supplements on metabolite production. During the study of antifungal production from Streptomyces violaceusniger, 70–90% enhancement in the yield was observed by supplementing xylose, sorbitol and hydroxyl proline in the production medium (Tripathi et al.

c) **Replacement experiments**-For medium formulation, carbon/nitrogen sources showing enhancement effect on the desired metabolite production in supplementation experiments are generally tried to be used as a whole carbon/nitrogen source.

4.2.2.Statistical medium optimization

With the advancement of statistical techniques, medium optimization has found new dimensions, as these techniques improve the efficiency of the process, reduces the time required in the process and labor cost etc., thus contributing toward the overall economics of the process.It can be perform by two ways **1)Screening of significant components,2)Optimization of component concentration**

1)Screening of significant components-This can be achive via different ways as given below:

a) ***Plakett burman design***

All the components present in the medium do not contribute in the metabolite production. Hence, it is utmost important that the non-contributing factors, should be eliminated from the study as early as possible. In 1946, R.L. Plackett and J.P. Burman published their work entitled "*The design of optimal multifactorial experiments*" as a solution to determine the major effects with higher precision in any process.

PDB is used to screen "*n*" number of experimental variables in just "*n*+1" number of experiments (Reddy et al.; Ghanem et al.). In this design, there are two types of variables, i.e., "*real variables*" whose concentration changes during the experiments, and "*dummy variables*," whose concentration remains constant during the experiments and used to estimate the error. Each variable is represented in two levels, i.e., high (H) and low (L). Each horizontal row represents a trial and each vertical column represents the either of two levels (high or low) of each independent and dummy variables in all the trials. Usually, the classical experiments help in the selection of independent and dummy variables. The effect of each variable is determined by the following equation:

Where, $E_{(X1)}$ = Effect of variable; $Y_{X1\text{-}H}$ = yield from the trials having high concentration of variable; Y_{X1-L} = yield from the trials having low concentration of variable and N = total number of trials.

b)***Taguchi design***

In order to overcome the problems associated with the PBD method, Dr. Genichi Taguchi developed a method which is based on "ORTHOGONAL ARRAY." This method tells us how different parameters affect the yield in a small number of experiments instead of testing all the possible combinations, like, the factorial design. Analysis of variance (ANOVA) on the collected data from the Taguchi DOE can be used to select the new parameter values to optimize the performance characteristic. During the execution of the experiment, at first the

total degree of freedom is selected [overall mean always uses 1 degree of freedom (DOF); for each factor DOF = n − 1, where n = number of levels; for any two factor interaction DOF = $(n_a - 1)(n_b - 1)$] followed by the selection of standard orthogonal array.

2)Optimization of component concentration- This can be achive via different ways as given below:

a) ***Response surface methodology (RSM)***

During the development of pharmaceutical formulations various production mediums and process variables related to the productivity, safety and usefulness should be optimized. Vaidya et al. used RSM for chitinase production from *Alealigenes xylosoxydans* and found 1.4-folds production enhancement.

RSM employs several phases of optimization (Gupte and Kulkarni) and it can be performed in three basic steps, i.e., experiments designed for the screening of the factors followed by the path of steepest ascent/descent and finally quadratic regression model is fitted and optimized using canonical regression analysis method. Since, the theoretical relationships between the independent and dependent variables are not clear, multiple regression analysis can be applied to predict the dependent variables on the basis of a second-order equation.

Where Y = predicted response, a_0 = intercept coefficient, a_iX_i = linear terms, $a_{ij}X_iX_j$ = interaction terms and $a_{ii}X^2$ = square terms.

b)***Genetic algorithm (GA)***

A trained mathematical model serves as a fitness function in the determination of optimum concentration of the medium components using GA. GA mimics the process of mutation and is based upon the principle “survival of the fittest”. This algorithm is based on the biological process of evolution, i.e., natural selection (Houck et al.).

The GA follows mainly three types of rules at each step to create the next generation from the current population: *Selection rule* selects the individuals, known as parents that contribute to the population of the next generation. *Crossover rule*combines two parents to form children for the next generation. *Mutation rule* applies random changes to individual parents to form children. GA was successfully used to optimize medium composition for rifamycin B production using mutant strain of *Amycolatopsis mediterranei* at shake flask level (Bapat and Wangikar).

5.REVIEW ON MEDIA OPTIMIZATION FOR CELLULASE PRODUCTION FROM *PENICILLIUM FUNICULOSUM*

Daniele Fernandes Carvalho el.al. optimized the media for cellulase production from *Penicillium funiculosum*. The present work aimed to optimise a bioprocess to produce these biocatalysts from the fungus *Penicillium funiculosum* ATCC11797. In particular, *P. funiculosum* ATCC 11797 has recently been identified as an outstanding source of well-balanced cellulolytic complexes .When used in the form of its enzyme extract, either alone or blended with *T. harzianum* cellulolytic extract, it has been shown to have great potential for biomass hydrolysis, yielding up to 86% cellulose conversion .

1)For carbon source optimisation they resuspended spores of *P. funiculosum* (5.33×10^7, total amount) were used to inoculate 100 mL of modified Mandels and Weber medium in 500 mL conic flasks, which were incubated at 30°C and 200 rpm for cell propagation. After 3 days,

10 mL of culture containing the growing cells (3.8 $g \cdot L^{-1}$) was transferred into 1 L Erlenmeyer flasks containing 200 mL of media containing supplemented with Avicel CE-15, medium viscosity carboxymethylcellulose (CMC), or cellobiose.

2)To obtain optimal composition of culture media, include urea, yeast extract , KH_2PO_4 and $MgSO_4 \cdot 7H_2O$,a full factorial design (2^4 FFD) was carried out to optimise the culture media composition.

3) To determine the best operational conditions for cellulase production, 1 L of optimised culture medium was inoculated in a 2 L stirred tank bioreactor. Agitation speed and aeration rate were considered to be the most important variables and used for a full factorial design (3^2 FFD).

They found that the optimal composition of culture media using Avicel (10 $g \cdot L^{-1}$) as carbon source was determined to include urea (1.2 $g \cdot L^{-1}$), yeast extract (1.0 $g \cdot L^{-1}$), KH_2PO_4 (6.0 $g \cdot L^{-1}$), and $MgSO_4 \cdot 7H_2O$ (1.2 $g \cdot L^{-1}$). The growth process was performed in batches in a bioreactor. Using a different FFD strategy, the optimised bioreactor operational conditions of an agitation speed of 220 rpm and aeration rate of 0.6 vvm allowed the obtainment of an enzyme pool with activities of 508 $U \cdot L^{-1}$ for FPase, 9,204 $U \cdot L^{-1}$ for endoglucanase, and 2,395 $U \cdot L^{-1}$ for *β*-glucosidase.

The present study aimed at maximizing cellulase production by Penicillium funiculosum using sequential experimental design methodology for optimizing the concentrations of nitrogen sources,conducted by Meada RN.They found that the optimal concentrations of urea and yeast extract predicted by the model were 0.97 and 0.36 g/L, respectively, which were validated experimentally. By the use of the desirability function, it was possible to maximize the three main enzyme activities simultaneously, which resulted in values for FPase of 227 U/L, for CMCase of 6,917 U/L, and for beta-glucosidase of 1,375 U/L. These values corresponded to increases of 3.3-, 3.2-, and 6.7-folds, respectively.

Another work conducted by R N Maeda & CA Barcelo,aimed to produce cellulase from *Penicillium funiculosum* by fed batch reactor. The enzyme production was performed in a bioreactor with two inoculum concentrations (5 and 10% v/v). The fermentation inoculated with higher inoculum size reduced the time for maximum enzyme production (from 72 to 48). The enzyme extract was concentrated using tangential ultrafiltration in hollow fiber membranes, and the produced cellulase blend was evaluated for its stability at 37 °C, operation temperature of the simultaneous SSF process, and at 50 °C, optimum temperature of cellulase blend activity. The cellulolytic preparation was stable for at least 300 h at both 37 °C and 50 °C.

6.DIFFERENT TYPES OF BIOREACTOR OPERATION & DESGN

6.1.Different mode of operation of homogeneous bioractor

A homogeneous bioreactor has been operated in four different way -1)batch operation,2)fed-batch operation,3)continuous operation &4)multi stage operation.

6.1.A.Batch Operation:Batch fermentation is initiated by inoculating a pre-sterilized and cooled medium. The reactor is a closed system and is highly dynamic in nature. Medium composition changes continuously with time to produce biomass and metabolites. The broth is harvested after the nutrients are consumed and products purified.Let us define t(l) as sum of the time required for the lag phase,harvesting & production phase.Thus total time to complete a batch cycle,t(c) is

$$t(c) = 1/\mu(m) \ln X(m)/X(0) + t(l)$$

where $X(m)$ = maximal attainable cell concentration & $X(0)$ = cell concentration at inoculation

The total amount of cell mass produced come from knowing the total amount of growth-extented-limit nutrient present & yield coefficient:

$$X(m)-X(0)=Y(x/s)\, S(0)$$

So The rate of cell mass production in a batch cycle:

$$r(b) = Y(x/s)S(0)/(1/\mu(m))\ln(X(m)/X(0))+t(l)$$

6.1.B. **Fed batch operation:** A fed batch cultivation is identical to a batch operation, except that a feed is added to the broth continuously or intermittently. The volume of the broth increases with time. Feed rate and inlet substrate concentration in fed batch cultivation is decided in such a way that it improves the product formation and productivity of the fermentation. Also nutrient feeding in the fed – batch cultivation should be so designed that when the reactor is full it is having high product concentrations and no unconverted substrate.

A fed batch system operates at quasi-steady state when nutrient consumption rate is nearly equal to nutrient feed rate.Since $dX/dt=0$ at quasi-steady state,then

$$\mu(net)=D$$

If maintenance energy can be neglected

$$\mu(net) =\mu(m)\, s/(K(s)+S)$$

$$S= K(S)D/(\mu(m)-D)$$

Product profile in a fed-batch culture can be obtained by using the definitions of $Y(P/S)$.When the product yield coefficient $Y(P/S)$ is constant,at quasi-steady state $S<<S(0)$

$$P=Y(P/S)S(0)$$

Or the potential product out put is:$FP=Y(P/S)S(0)F$

Continuous reactors: fresh media is continuously added and bioreactor fluid is continuously removed. As a result, cells continuously receive fresh medium and products and waste products and cells are continuously removed for processing. The reactor can thus be operated for long periods of time without having to be shut down. Continuous reactors can be many times more productive than batch reactors. This is partly due to the fact that the reactor does not have to be shut down as regularly and also due to the fact that the growth rate of the bacteria in the reactor can be more easily controlled and optimized. In addition, cells can also be immobilized in continuous reactors, to prevent their removal and thus further increase the productivity of these reactors.

7.REVIEW ON VOLUMETRIC EFFECT ON MICROBIAL GROWTH & PRODUCTIVITY:

Microbial batch growth during confined incubation in bottles of various sizes is used daily in a broad variety of microbiological studies and methods, including bioassays such as the assimilable organic carbon (AOC) assay and the analysis of pure culture or microbial community growth in freshwater.This phenomena is termed as volumetric effect.

The uncertainty surrounding this apparent effect was clearly summarized in a recent paper by Pernthaler and Amann : "Such investigations are often plagued by the mysterious 'bottle effect', a hard-to-define concept that reflects the worry of whether phenomena observed in confined assemblages are nonspecific consequences of the confinement rather than a result of the planned manipulation."

Claude E Zobell & D Quentin Anderson found that storage of sea water is accomplished by great increase in the number of bacteria & decrease in bactia species.The favourable influence in small volume was attributed to contact of the water to larger solid surface in a small receptacles.Between ten & hundred million bacteria per cc has been demonstated in the sea water store in the sand which present in inormous surface area ,whethere less than three hundred thousand per cc appearing similar water stored in a 10 litre bottle.

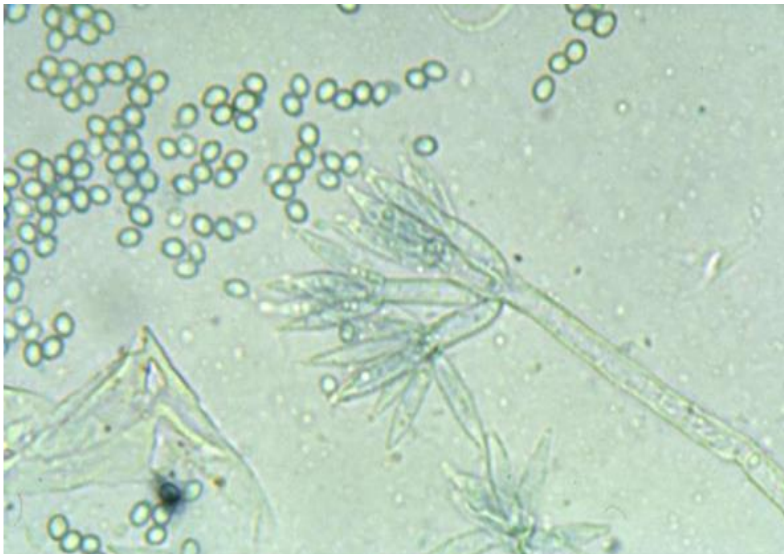
Frederik Hammes , Marius Vital and Thomas Egli analysed the impact of surface-to-volume ratio on final bacterial concentrations after batch growth. We examined six bottle sizes (20 to 1,000 ml) using three independent enumeration methods to quantify growth.
Three batch growth experiments were conducted to assess the volumetric bottle effect on final cell concentrations after growth into stationary phase. Six different bottle sizes were used, covering the ranges most often reported in "bottle effect" literature.The bottle sizes were as follows (water volumes and surface area-to-volume ratios [square centimeters to milliliters] are respectively included in parentheses): 1,000 ml (900 ml, 0.3:1), 500 ml (400 ml, 0.4:1), 250 ml (200 ml, 0.6:1), 100 ml (90 ml, 0.8:1), 40 ml (35 ml, 1.5:1), and 20 ml (15 ml, 2.4:1).
1)In the first experiment, a sample of natural river water (dissolved organic carbon [DOC], 3.8 mg/liter; AOC, 0.3 mg/liter) from a small oligotrophic stream was obtained, filter sterilized with a 50-kDa dialysis filter (Fresenius Medical Care), and inoculated (at 10^3 cells/ml) with a microbial community used for AOC assays.
2)In the second experiment, a sample of the effluent (DOC, 1.2 mg/liter; AOC, 0.03 mg/liter; total cell concentration [TCC], 3×10^5 cells/ml) from a granulated active carbon filter situated.
3)or the third experiment, sterile Luria-Bertani (LB) medium (diluted 1:10,000; DOC, 0.7 mg/liter; AOC, 0.46 mg/liter) was inoculated with *Vibrio cholerae* O1 (10^3cells/ml) .
In conclusion, we did not observe evidence of a volumetric bottle effect on short-term (<5-day) batch incubations. The findings of this study suggest that reference to the so-called volumetric bottle effect should be considered carefully unless supported by clear experimental data.

Bischofberger et al.observed that incubation of groundwater led to significantly more growth (about 2 log units) in small bottles (100 ml) than in big ones (10 liters).

8.*Penicillium funiculosum*

Penicillium funiculosum strains have been reported as producers of cellulolytic complexes with improved synergy due to their high production of β-glucosidase and endoglucanase . In particular, *P. funiculosum* has recently been identified as an outstanding source of well-balanced cellulolytic complexes . When used in the form of its enzyme extract, either alone or

blended with *T. harzianum* cellulolytic extract, it has been shown to have great potential for biomass hydrolysis, yielding up to 86% cellulose conversion . Although *P. funiculosum* can efficiently produce cellulases from pretreated agroindustrial residues , there is a great variability in the source and composition of such materials that negatively affects the reproducibility of results obtained under optimised conditions. The present study investigated the optimisation of culture conditions for cellulase production by *Penicillium funiculosum* . This organism was grown by submerged fermentation using certified model carbon sources as substrates, and the culture medium composition and operational conditions were modified with the aim of maximising the rate of enzyme production. The growth of *P. funiculosum* can occur from 8 to 42°C with an optimum at 25-28°C. This species does not live through a 30-minute exposure at 70°C.Acid-tolerant species able to develop on acidic soils at pH 2.



9.REVIEW ON cre A MUTATION:

Carbon catabolite repression (CCR): Carbon catabolite repression (CCR) is an important mechanism to repress the production of plant cell wall degrading enzymes during growth on preferred carbon sources. In addition to regulation by CCR, production of hydrolytic enzymes associated with plant cell wall degradation is induced to high levels only in the presence of plant cell wall biopolymers or their derivatives.

Some aspects of CCR are mediated by Mig1/CreA/CRE1, a zinc-finger transcription factor conserved in most fungal specie. In *Saccharomyces cerevisiae*, under conditions of glucose sufficiency, Mig1 functions to repress transcription of ~90 genes associated with utilization of alternative carbons sources, such as maltose, galactose and sucrose. In *Aspergilli* and *H. jecorina*, CreA/CRE1 was shown to directly regulate genes involved in xylose, xylan, arabinose, proline and ethanol utilization, as well as penicillin biosynthesis (; the binding motif of CreA was shown to be 5′-SYGGRG-3′and is context dependent . CreA is believed to regulate the transcription of genes in a "double-lock" manner.

Deletion of *cre-1* increased cellulolytic enzyme production: In *N. crassa*, the Δ*cre-1* mutant grows slower and denser than wildtype (WT) when grown on preferred carbon sources, such as glucose, sucrose or xylose, similar to the phenotype of *A. niger* and *T. reesei creA/cre1* mutants . However, no differences in growth rate or morphology from a WT strain were observed when Δ*cre-1* was grown on carboxymethylcellulose (CMC), glycerol or sodium acetate (NaAc) media . When grown on 2% Avicel medium as a sole carbon source, the Δ*cre-1* strain consumed Avicel faster than WT (*e.g.* 3–4 days vs 5–6 days), secreted 30% more extracellular protein and showed 50% higher endoglucanase activity. An aggregate Avicelase assay (which measures combined β-glucosidase, endo-, and exo-cellulase activity) showed 20% higher glucose concentrations in the Δ*cre-1* strain as compared to WT.

Diverse Regulation of the CreA Carbon Catabolite Repressor in *Aspergillus nidulans:*

In the filamentous fungus *Aspergillus nidulans*, CCR is mediated by the transcription factor CreA, a C_2H_2 finger domain DNA-binding protein. The aim of this work was to investigate the regulation of CreA and characterize its functionally distinct protein domain.

Similar to the study carried out in *T. reesei*, *A. nidulans*CreA depends in part on *de novo* protein synthesis. Microscopic studies and Western blots detected the CreA::GFP protein in cell extracts treated with the protein synthesis inhibitor cycloheximide. Accordingly, the expression of a CreA::Luc strain in the presence of xylanase- and cellulase-repressing and -inducing carbon sources showed major fluctuations in CreA protein levels, but the expression of CreA remained relatively high under all tested conditions, including nonglucose complex carbon sources. The readily available CreA protein pools therefore would allow the fungus to quickly adjust gene expression and metabolism once glucose is detected.

Part-4

Materials & Methods

1. MATERIAL & METHODS FOR CELLULASE ASSAY

1. a. Standard Operating Protocol for FPU

1. a.1. **Purpose**

This SOP describes the procedure for FPA (filter paper assay) the most common total cellulase activity assay recommended by the International Union of Pure and Applied Chemistry (IUPAC). This assay is based on a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper (i.e., both amorphous and crystalline fractions of the substrate are hydrolyzed) within a fixed time (i.e., 60 min). Total cellulase activity is described in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. The strengths of this assay are that (1) the substrate is widely available and (2) the substrate is reasonably susceptible to cellulase activity.

Compliance with this SOP is of importance because it allows the clear understanding and consistent reproduction of the established laboratory procedures by the person (s) performing the task.

This SOP provides instructions and support for performing the enzyme assays at three bioenergy centres. This document applies to all employees who are authorized and trained to use this procedure.

1.a.2. Materials

1) Whatman No.1 filter paper

2) Citric acid monohydrate (ACS reagent)

3) Sodium hydroxide pellets

4) 3,5-Dinitrosalicylic acid – Sigma

5) Rochelle salts (Na-K tartarate) – Himedia

6) Phenol – Sigma

7) Sodiummetabisulfite ($Na_2S_2O_5$) – Sigma

8) D-(+)-Glucose – Sigma

9) Deionized purified water, laboratory supply

1. a. 3. Solution Preparations:

0.1M Citrate Buffer of pH=4.7,

To prepare 100ml of 0.1M Citrate Buffer, 25.5 ml of 0.1 M citric acid was mixed with 24.5ml ,0.1 M of sodium citrate & then remaining volume was make up with 50 ml water & p H was adjusted about 4.7.Futher dilution was made from this stock solution

Dinitrosalicylic acid (DNS Reagent preparation inside dark bottle)

283.2 ml of deionized water is measured, put in dark bottle and 2.12 g of 3,5-Dinitrosalicylic acid (DNS) and 3.96 g of NaOH were added, keep on stirrer until all these mix well.
Now 61.2 g of Rochelle salts (Na-K tartarte), 1.52 ml of phenol and 1.66 g of Sodium Metabisulphite were added in the solution. Keep on stirrer until everything is dissolved in side dark bottle. Store the DNS reagent at 4℃ until two weeks.

1.a.4. **Substrate**
Whatman No. 1 filter paper strip was cut exactly into 1.0 x 6.0 cm (= 50 mg) dimensions.

1.a.5.Instruments and Equipment

1) Constant Temperature Water Bath at 50.0°C ± 0.5°C.

2)Boiling Water Bath approximately 100°C.

3)pH Meter

4)Spectrophotometer ultrospec 3100 Pro, Amersham Biosciences

5)Laboratory Stirrer/Hotplate

6)Pipetman, 2 µl, Gilson or equivalent

7)Pipetman, 20 µl, Gilson or equivalent

8)Pipetman, 200 µl, Gilson, or equivalent

9)Pipetman, 1000 µl, Gilson or equivalent

10)Disposable micro liter pipette tips in racks, 1 ml

11)Disposable micro liter pipette tips in racks, 200 µl,

12)Test tubes of at least volume 25 ml,

13)Magnetic stir bars

14)Aluminum foil, or equivalent

15)Measuring Cylinders, 100 ml, 500 ml, 1000 ml

16)Disposable vinyl gloves

17)Timer suitable for timing 20 min to 2 hour intervals

1.a.6. Procedure

1.1.0 ml 0.05 M citrate buffer, pH 5.0 is added to a test tube of volume at least 60 ml.

2. 0.5 ml enzyme, diluted is added in citrate buffer. At least three dilutions must be made of each enzyme sample investigated.

3. One filter paper strip is added & mixed (NB! it does not matter if a small part of the paper- i above the liquid surface, but if the paper "winds" up the tube it must be pushed down again)

4. The above test tubes are incubated at 50°C for 60 min.

5. Blanks and controls:

5.1 Spectro zero: 1.5 ml citrate buffer (use this solution to set zero in spectro)

5.2 Substrate blank: 1.0 ml citrate buffer + 0.5 ml enzyme dilution (prepare a separate

control for each dilution tested).

5.3 Enzyme blank: 1.5 ml citrate buffer + filter paper strip

.6. After incubation, test tubes were chilled the tube in ice water for 5 min

7. 3.0 ml DNS was added & mixed & then Transferred to a rack on the table.

8. Then test tubes were boiled for exactly 5.0 min in a vigorously boiling water bath containing sufficient water.

All samples, enzyme blanks, substrate blank, glucose standards and the spectro zero should be boiled together. After boiling, transfer to ice water bath for 5 min.

9. 50 ml deionized was added water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion

(NB. This is important!).

10. When the 'pulp' has settled well, i.e., after at least 10 min, the colour formed was measured against the spectro zero at 540 nm

1.a.7. Glucose Standard Preparation

1.a.7.1.Glucose Stock solution 10mg/ml anhydrous glucose

Dilutions are made from the stock solution (10 mg/ml) in the following manner:

1.0 ml of glucose stock + 0.5 ml buffer = 1:1.5 = 6.7 mg/ml (3.35 mg/0.5 ml)
1.0 ml of glucose stock + 1.0 ml buffer = 1:2 = 5.0 mg/ml (2.5 mg/0.5 ml)
1.0 ml of glucose stock + 1.5 ml buffer = 1:2.5 = 4.0 mg/ml (2.0 mg/0.5ml)
1.0 ml of glucose stock + 2.0 ml buffer = 1:3 = 3.3 mg/ml (1.65 mg/0.5 ml)
1.0 ml of glucose stock + 4.0 ml buffer = 1:5 = 2.0 mg/ml (1.0 mg/0.5 ml)

1.b. SOP For CMC(Endo-β-1,4-D-glucanase (EC 3.2.1.4)) Assay

1.b.1.Purpose

This SOP describes the procedure for Endo-β-1,4-D-glucanase (EC 3.2.1.4) which randomly cleaves accessible intermolecular β-1,4-glucosidic bonds on the surface of cellulose. Water-soluble cellulose derivatives such as CMC are commonly used for endoglucanase activity assays. The hydrolysis can be determined by measuring the reducing sugars. The purpose of the Endoglucanase assay is to detect and evaluate the endoglucanase activity in the sample.

Compliance with this SOP is of importance because it allows the clear understanding and consistent reproduction of the established laboratory procedures by the person (s) performing the task.

This SOP provides instructions and support for performing the enzyme assays at three bioenergy centres. This document applies to all employees who are authorized and trained to use this procedure.

1.b.2. Materials

1)Carboxy methyl cellulose sodium salt – Sigma

2)Citric acid monohydrate (ACS reagent) – Sigma

3)Sodium hydroxide pellets – Merck

4)3,5-Dinitrosalicylic acid – Sigma Cat.

5)Rochelle salts (Na-K tartarate) – Himedia

6)Phenol – Sigma

7)Sodiummetabisulfite ($Na_2S_2O_5$) – Sigma

8)D-(+)-Glucose – Sigma

9)Deionized purified water, laboratory supply

1.b.3.Solution Preparations:

0.1 M citrate buffer of p H 4.7 was prepared in similar procedure as 1.a.3
Dinitrosalicylic acid (DNS Reagent preparation inside dark bottle)

DNS reagent also prepared in similar procedure as mentioned in 1.a.4.

1.b.4.CMC Substrate Solution (2%)

To make 50 mL of substrate solution 1 g of CMC was dissolvedin 45 ml deionized water and 5 ml of 1M citrate buffer(pH5.0) was added.

1.b.5. Instruments and Equipment

1)Flat bottom Microtiter plates, 96 well - Costar Cat. No. 9018

2)Microplate reader, Spectramax M3 Molecular Devices

3)Spectophotometerultrospec 3100 Pro, Amersham Biosciences

4)Laboratory Stirrer/Hotplate

5)Pipetman, 2 ul, Gilson or equivalent

6)Pipetman, 20 ul, Gilson or equivalent

7)Pipetman, 200 ul, Gilson, or equivalent

8)Pipetman, 1000 ul, Gilson or equivalent

9)Disposable microliter pipette tips in racks,1ml

10)Disposable microliter pipette tips in racks, 200 μl,

11)Eppendorf safe-lock 1.5 ml tubes,

12)Magnetic stir bars

13)Aluminum foil, or equivalent

14)Measuring Cylinders, 100mL, 500mL,1000mL

15)Disposable vinyl gloves

16)Timer suitable for timing 20 min to 2 hour intervals

1.b.6.Procedure

Methodology for small volume adaptable to micro-titer plate (screening):

1.Each reaction should be set-up in triplicate

2.0.25ml of enzyme was added in 1.5 ml microfuge tube or deep-well plate

3. 0.25ml of 2% CMC was added in 0.01M citrate-phosphate buffer (pH 5.0)

4.Mix well and incubate at 50℃ for 30 min

5.0.5ml of DNSA, was added mix well

6.Blank and controls:

6.1.Substrate blank: 0.25 ml citrate buffer + 0.25 ml enzyme

6.2.Enzyme blank: 0.25 ml citrate buffer + 0.25 ml of 2% CMC.

6.3.Boil for 10 min at 100℃. All samples, enzyme blank, substrate blank, glucose standards should be boiled together.

7.100μl of above reaction was transferred in 96-well micro-titer plate.

8.The absorbance was measured at 540nm on micro-titer plate reader.

1.b.7. Preparation of Glucose standard:

Glucose Stock solution 1mg/ml (micro-titer plate)

Dilutions are made from the stock solution (1mg/ml) in the following manner:

1.0 ml of stock solution + 15.0 ml of 50mM buffer = 0.0625 mg/ml

1.0 ml of stock solution + 7.0 ml of 50mM buffer =0.125 mg/ml

1.0 ml of stock solution + 3.0 ml of 50mM buffer = 0.25 mg/ml

1.0 ml of stock solution + 1.0 ml of 50mM buffer = 0.5 mg/ml

Undiluted = 1 mg/ml

1.c. SOP For Xylanase assay:

1.c.1.Purpose

This SOP describes the procedure for Xylanase (endo-1,4-ß-D-xylan; EC 3.2.1.8) which hydrolyses the xylan (units of xylose, pentose sugar).Historically, xylanase activity has been measured with the DNS reducing sugar assay (Bailey, 1988) with purified xylan from oat spelts, larchwood or birchwood as substrate.The purpose of the xylanase assay is to detect and evaluate the xylanase activity in the sample.

Compliance with this SOP is of importance because it allows the clear understanding and consistent reproduction of the established laboratory procedures by the person (s) performing the task.

This SOP provides instructions and support for performing the enzyme assays at three bioenergy centres. This document applies to all employees who are authorized and trained to use this procedure.

1.c.2.Materials

1)Xylan from oat spelts – Himedia

2)Citric acid monohydrate (ACS reagent) – Sigma

3)Sodium hydroxide pellets – Merck

4)3,5-Dinitrosalicylic acid – Sigma

5)Rochelle salts (Na-K tartarate) – Himedia

6)Phenol – Sigma

7)Sodiummetabisulfite ($Na_2S_2O_5$) – Sigma

8)D-(+)-Xylose – Sigma

9)Deionized purified water, laboratory supply

1.c.3. Solution Preparations:

0.1 M citrate buffer of p H 4.7 was prepared in similar procedure as 1.a.3

Dinitrosalicylic acid (DNS Reagent preparation inside dark bottle)

DNS reagent also prepared in similar procedure as mentioned in 1.a.4.

1.c.4.Xylan Substrate Solution (2%)

To make 50 ml of 2% Xylan substrate solutio 1 g of xylan was dissolved in 45 ml deionized water and 5 ml of 1 M citrate buffer (pH 5.0) was added.

1.c.5.Instruments and Equipment

1)Flat bottom Microtiter plates, 96 well - Costar Cat. No. 9018

2)Microplate reader, Spectramax M3 Molecular Devices

3)Spectophotometerultrospec 3100 Pro, Amersham Biosciences

4)Laboratory Stirrer/Hotplate

5)Pipetman, 2 µl, Gilson or equivalent

6)Pipetman, 20 µl, Gilson or equivalent

7)Pipetman, 200 µl, Gilson, or equivalent

8)Pipetman, 1000 µl, Gilson or equivalent

9)Disposable micro liter pipette tips in racks, 1 ml

10)Disposable micro liter pipette tips in racks, 200 µl,

11)Eppendorf safe-lock 1.5 ml tubes,

12)Magnetic stir bars

13)Aluminum foil, or equivalent

14)Measuring Cylinders, 100 ml, 500 ml, 1000 ml

15)Disposable vinyl gloves

16)Timer suitable for timing 20 min to 2 hour intervals

1.c.6.Procedure

Methodology for small volume adaptable to micro-titer plate (screening):

1.Each reaction should be set-up in triplicate

2.0.25 ml of enzyme was added in 1.5 ml microfuge tube or deep-well plate

3. 0.25 ml of xylan was added substrate solution in the tube or plate

4.Mix well and incubate at 50 ℃ for 30 min

5.Blanks and controls:

5.1.Substrate blank: 0.25 ml citrate buffer + 0.25 ml enzyme

5.2.Enzyme blank: 0.25 ml citrate buffer +0.25 ml 2% xylan substrate solution

6. 0.5 ml of DNS was added mix well

7. All samples, enzyme blank, substrate blank, xylose standards were boiled together for 10 min at100 ℃.

8. 100 µl of above reaction was transferred in 96-well micro-titer plate

9.The absorbance was at 540 nm on micro-titer plate reader.

1.c.7.Preparation of xylose standard:

Xylose Stock solution 1mg/ml (micro-titer plate)

Dilutions are made from the stock solution (1mg/ml) in the following manner:

1.0 ml of stock solution + 19 ml of 50 mM buffer = 0.05 mg/ml

1.0 ml of stock solution + 9.0 ml of 50mM buffer = 0.1 mg/ml

1.0 ml of stock solution + 5.6 ml of 50mM buffer = 0.15 mg/ml

1.0 ml of stock solution + 4.0 ml of 50mM buffer = 0.20 mg/ml

1.0 ml of stock solution + 3.0 ml of 50mM buffer = 0.25 mg/ml

1.0 ml of stock solution + 2.3 ml of 50mM buffer = 0.30 mg/ml

2.MATERIALS& METHOD FOR SPORULATION MEDIA OF *Penicillium funiculosum*

2.1.Sporulation media composition:

Component	gm/litre
1)Lactose	15
2)Corn steep liquor	6.5
3)Peptone	5.0
4)Nacl	4.5
5)Magnesiumsulphate	0.50
6)Potassiumdihydrogen Phosphate	0.72
7)Ferric chloride	0.006
8)Coppersulphate	0.002
9)Agar `	50.00

The above media p H is adjust within 4.8 to 5.0.And after autoclaving 30ml of media is taken in 250ml conical flask.When the media is solidified then 5ml of spore of *Penicillium funiculosum* is added on the upper surface and its gently spreaded by sterile speader in laminar air hood.

3. MATERIAL& METHOD FOR VOLUMETRIC EFFECT ON CELLULASE PRODUCTION & ACTIVITY BY *P. funiculosum* WILD TYPE& MEDIA OPTIMIZATION

3.1.Principle: Media optimization is a process through which different condition & components of the media are varied or changed so that we can get the optimum growth of micro organism& optimum productivity. This can be achieved by either ‘one factor at a time’ or by modern statistical and mathematical method.

a)Rich media- A rich media is a type of complex media that contain rich nutrients and water soluble extract of plant or animal tissue such as enzymatically digested animal protein like tryptone or peptone. Generally glucose is added as a carbon source.

b) Minimalmedia-A minimal media contain minimum nutrients possible for growth of microorganism with small amount of nitrogen source or amino acid or without any amino acid. This type of media is mainly used to grow wild type as well as recombinant. A carbon source which may be glucose or less energy rich succinate may be used. In minimal media microorganism generally provided with essential salt like magnesium, sulfur, phosphorous.

Volumetric effect-Microbial growth and secondary metabolite or extra cellular enzyme productivity during incubation in liquid media is effected by surface to volume ratio, termed as volumetric effect.

3.2.1.Composition of R. C .Medium:

Components	gm/l
1)Soya peptone	24
2) Potassium dihydrogen Phosphate(KH_2PO_4)	5.9
3)Ammonium bi sulphate,$(NH_4)SO_4$	3.12
4)Calcium chloride dehydrate,$CaCl_{2,}2H_2O$	0.05
5)Yeast extract	0.05
6)Wheat bran	24
7)Avicel	21.4

p H-5.5

3.2.2.Composition of Maendael's Minnimum Medium:

Component	gm/litre
1)Avicel	25
2)Wheat bran	25
3)Calcium Chloride dehydrate	0.3
4)Potassium phosphate monobasic	2
5)Urea	0.3
6)Megnisiumsulphatehetahydrate	0.3

7)Peptone	0.25
8)Yeast extract	0.1
9)Tween 80	1 ml
10)Di ammonium sulphate	1.4
After autoclave	
11)Ferric sulphate heptahydrate	0.005
12)Manganase sulphate monohydrate	0.0016
13)Zinc sulphate heptahydrate	0.0014
14)Cobalt chloride hexahydrate	0.002

3.3.Procedure to study volumetric effect on cellulase production from *Penicillium funiculosum* wild type in 1:5, media:flask volume:

1) Initially 10^7 spore/ml was added in potato dextrose broth and allow to growfor 24 hours at 28°C in incubator.

2)After 1 day incubation 5% of inoculum is addedto each medium in each volumes.

3)Two volumes were choosen for this 1:5,medium:flask volume study.

A) 50 ml medium of each RCM & MM was taken in 250ml flask.

B) 200ml medium of each RCM & MM was taken in 1 litre flask.

4) Each flasks were set in biological triplicate.

5)The samples were collected in each 24 hours interval from 1st day to 7th day.

6)All samples are centrifuged at 4°C ,8500 rpm for 15 mins.

7) FPU,CMCS,Xylanase& SDS PAGE are performed to analyse cellobiohydrolase,endo-beta-1-4-D-glucanase,endo-1,4-beta-D-xylan EC3.2.1.8 or xylanase assay and to check cellulase production over the days as mentioned.

4.MATERIALS & METHOD FOR PACKED CELL VOLUME OPTIMIZATION & DRY CELL WEIGHT ANALYSIS OF *P. funicunosum* WILD TYPE AT DIFFERENT SPORE CONDITION

4.1 Principle: This is proportion of volume occupied by cell in a cell culture media. Cell volume determined by sedimenting cell using centrifugation. So packed cell volume is given by

Packed Cell Volume=Total volume of media – Volume of supernatant after centrifugation

4.2.Materials required:

1) Potato Dextrose Broth

2) Auloclaved water

3) Penicillium funiculosum spore $6*10^7$

4.3. Equipment required:

1) Conical flask of 250ml volume

2) Incubator set at 30°C

3) Pipetman, 0.2-2 µl, Gilson or equivalent

4) Pipetman, 2-20 µl, Gilson or equivalent

5)Pipetman, 20-200 µl, Gilson, or equivalent

6) Pipetman, 100-1000 µl, Gilson or equivalent

7) Hemocytometer

8) Microscope

4.4. Procedure:

1) I have considered three spore concentration of *Penicillium funiculosum* 10^5 spore/ml,10^6 spore/ml and 10^7 spore/ml .I also observed packed cell volume and dry weight in 24 hours interval and 48 hours intervals.

2) I have taken 1:10, media:flask volume ratio. So I have taken 25 ml of medium in 250 ml conical flask.

3)Dilutions are made in following manner:

3.1.For 10^7spore/mlconcentration=4.167ml of $6*10^7$spore/ml+12.5 ml autoclaved PDB media+8.33 ml of autoclaved R.O water.

3.2.For 10^6 spore/ml concentration=0.417 ml of $6*10^7$ spore/ml+12.5 ml of autoclaved PDB media+12.083 ml of auloclaved water

3.3.For 10^5 spore/ml concentration=0.04167 ml of $6*10^7$spore/ml+12.5 ml of autoclaved PDB media+12.45 ml of autoclaved R.O water

4) All inoculation set in biological duplicate.After inoculation flasks are kept in 30 °Cincubator.

5) The sample are taken in 50ml falcon after 24 and 48 hours interval .All the sample are centrifuged at 8500 rpm for 30 mins.

6) After centrifugation the supernatant is measured and pellets are kept in 80 °C hot air oven for 8 hours to evaporator water and to measured dry weight.

Note: Total volume of PDBmedium required 400ml.And molecular weight of PDB is 24gm,so for 400ml 9.6 gm of PBD media required.I have diluted 9.6 gm of PDB in 200ml water and remaining 200 ml of water is autoclaved for volume makeup purpose as given above.

5. MATERIALS& METHODS FOR INDUSTRIAL SCALE PRODUCTION OF CELLULASE FROM WILD TYPE OF *PENICILLIUM FUNICUNOSUM*IN 20 LITRE STIRRED TANK BIOREACTOR/FERMENTOR

5.1. Principle: Continuous stirred tank reactor is traditional bioreactor use in chemical industry & fermentation industry which run at steady state with continuous flow of product & reactant

5.2. Composition& preparation of primary, secondary& tertiary medium:

5.2.1. Composition of primary medium:

To prepare 200ml of primary medium 4.8 gm of PDB is dilute in 200ml of R.O water.

5.2.2. Composition & preparation of secondary medium:

The total volume of secondary medium is 1250ml and composition given below:

Component	**gm**
Glucose	31.25
Soya peptone	2
Di ammonium sulphate	3.9
Calsium chloride dehydrated	0.0625

Yeast extract	0.0625
Potassium phosphate monobasic	7.37
Corn steep liquir	12.5

5.2.3. Composition& preparation of tertiary medium:

The total volume of tertiary medium for 20 litre fermentor is 14 litre:

Component	**gm**
Soya peptone	224
Potassium phosphate monobasic	82.6
Di ammonium sulphate	43.68
Calsium chloride dehydrated	0.7
Yeast extract	0.7
Corn steep liquir	140ml
Micro crystalline cellulose	299
Wheat bran	336

5.3. Preparation of Acid, Base& Antifoaming agent:

5.3.1. Preparation of 40% of HCL

To make 700ml of 40% of HCL 280 ml of 37% of HCL is added in 420 ml of R.O water.

5.3.2. Preparation of 10(N) NaOH :

To prepared 700ml of 10(N) of NaOH 280 gm of NaOHis added to 700ml of R.O water with continuous stirring.

5.3.3.Anti foaming agent:

500ml of antifoaming agent is autoclaved for fermentor use.

5.3.4.Instrument& Equipment:

5.3.5.Procedure:

1)For fermentor run with wildtype of *Penicilliumfunicunosum* for cellulase production mainly

three type of media are set primary,secondary& tertiary media.Primary media was made of potato dextrose broth about 25 ml in 250ml conocal flask to matain surface to volume ratio 1:10.

2) To inoculate with 10^6 spore/ml concentration inoculation was made in following manner:

12.5ml PDB media+0.52ml of spore of Penicilliumfunicunosum from stock $4.8*10^7$ spore/ml & incubated for 48 hours at 30º c.

3) After 48 hours incubation 20 ml of primary media was added to the secondary media of 200ml media set in 2 litre flask to provide 1:10 volume to surface ratio & incubate it for 48 hours.

4) To run the 20 litre continuous stirred tank reactor or fermentor with tertiary media & secondary culture as a inoculum first fermentor was autoclaved with 14 litra water that consisting following phase:

Phase 1- First temperature was rais at 85º c& then further to 121º c& sterilization took place for 5 mins.

Phase 2 – Then temperature was decreased first to 80º c& then 55º c followed by 37º& then sterilization was stoped.

After autoclaved with water fermentor was auloclaved with 14 litre tertiary media (Modified RCM media) & as it came to 37º c at end of cooling phase of sterilization process leave it for 12 hous.

5)Then 1200ml of secondary inoculum is fed to tertiary media with pump through sterile pipe with continouous agitation at 200 rpm.

6)Then 40% of HCL of 800ml volume, 10(N) NaOH solution of 800 ml volume & 500ml antifoaming agent were fed by pump.

7)30º C ,temperature,30% of dissolve oxygen,200 rpm rotor speed & p H 5 were maintained as temperature,rpm& dissolved oxygen were controlled automatically but p H was maintained manually.

8)Sample was taken in each 24 hours interval about 50ml volume up to 9th day.

9)Then CMCS & FPU assay & SDS PAGE were done with all the sample to analyse all sample activity & packed cell volume was measured.

6. MATERIALS & METHODS FOR INDUSTRIAL SCALE PRODUCTION OF CELLULASE FROM cre A MUTANT STRAIN OF *PENICILLIUM FUNICULOSUM* IN 21 LITRE FERMENTOR

6.1. Principle,primary,secondary & tertiary media composition were similar as of wild type fermentor run mentioned in 5.1,5.2 & 5.3.

6.2. Procedure : **1)**For fermentor run with wildtype of *Penicilliumfunicunosum* for cellulase production mainly three type of media are set primary,secondary& tertiary media.Primary media was made of potato dextrose broth about 25 ml in 250ml conocal flask to matain surface to volume ratio 1:10.

2) Stock spore concentration of cre A mutant was 5* 10^7spore/ ml concentration.To inoculate with 10^6 spore/ml concentration inoculation was made in following manner:

12.5 ml of PDB + 0.5 ml of 5*10^7 spore/ml spore + 12 ml of RO water

3) After 48 hours incubation 20 ml of primary media was added to the secondary media of 200ml media set in 2 litre flask to provide 1:10 volume to surface ratio & incubate it for 48

hours.

4) To run the 20 litre continuous stirred tank reactor or fermentor with tertiary media & secondary culture as a inoculum first fermentor was autoclaved with 14 litra water that consisting following phase:

Phase 1- First temperature was rais at 85º c& then further to 121º c& sterilization took place for 5 mins.

Phase 2 – Then temperature was decreased first to 80º c& then 55º c followed by 37º& then sterilization was stoped.

After autoclaved with water fermentor was auloclaved with 14 litre tertiary media (Modified RCM media) & as it came to 37º c at end of cooling phase of sterilization process leave it for 12 hous.

5)Then 1200ml of secondary inoculum is fed to tertiary media with pump through sterile pipe with continouous agitation at 200 rpm.

6)Then 40% of HCL of 800ml volume, 10(N) NaOH solution of 800 ml volume & 500ml antifoaming agent were fed by pump.

7)30º C ,temperature,30% of dissolve oxygen,200 rpm rotor speed & p H 5 were maintained as temperature,rpm& dissolved oxygen were controlled automatically but p H was maintained manually.

8)Sample was taken in each 24 hours interval about 50ml volume up to 9th day.

9)Then CMCS & FPU assay were done with all the sample to analyse all sample activity & packed cell volume was measured.

Part -5
Result & Discussion

1.RESULT OF MEDIA SELECTION & OPTIMISATION IN 1:5 VOLUME-TO-SURFACE RATIO OF 50ML & 200ML VOLUME IN SMALL SCALE:

In this analysis to optimise the cellulase production from *Penicillium funiculosum* wild type in small scale following results were obtained

For optimisation & selection of the media & also study the 1:5 volume:surface ratio in two volume 50ml & 200 ml,it had been seen that greater production of endogluconase enzyme in RCM 50 ml volume rather than 200 ml.And optimum production of endogluconase was occurred on 7th for both cases (figure-1.1).It had been seen greater production of endogluconase in MM 50ml volume rather than MM 200ml volume & optimum production of endogluconase had been seen on 7th day in both cases (figure-1.2) .Figure 1.3 greater production of endogluconase in RCM media in both volume than MM media. In figure-1.5 it had been seen that same type of production pattern of xylanase(endo-1,4-ß-D-xylan; EC 3.2.1) in MM media both 50ml & 200ml volume & xylanase production was optimum on 6th day &5th day in MM 50ml & 60ml volume respectively.

In figure -1.6 it had been seen same type of production pattern of xylanase(endo-1,4-ß-D-xylan; EC 3.2.1) in RCM media both 50ml, 200ml volume,MM 50ml & MM200ml.

In figure-1.7 it had been seen that FPU unit was greater on 6th day than 5th day in all cases i.e.RCM 50ml,RCM 200ml,MM 50ml & MM 200ml.And RCM 50 ml shown greater FPU

Table-1.1.Result of CMC assay of RCM 50ml & RCM 200ml:

DAY	RCM 50ml,IU/ML	RCM 200ml,IU/ML
3	20.3362	6.983558
4	19.6687	14.07142
5	15.50037	12.60833
6	13.62576	11.57953
7	19.93514	21.20195

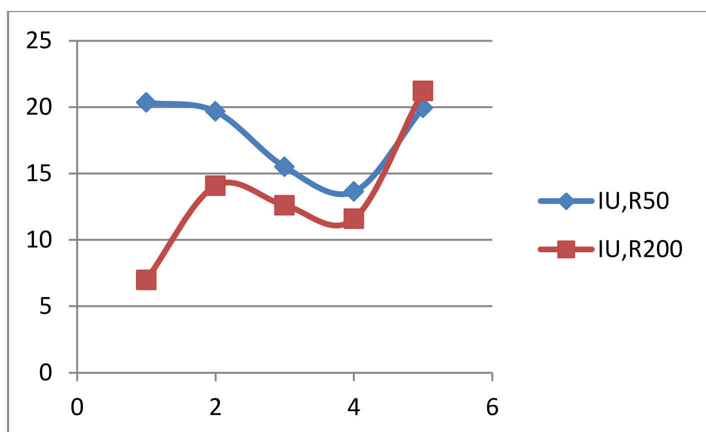


Figure-1.1-CMC assay of RCM50ml & RCM200ml

1.2.Result of CMC assay of MM 50ml & MM 200ml:

DAY	MM 50ml,IU/ML	MM 200ml,IU/ML
3	5.364451	1.767123
4	5.842212	5.948757
5	7.043	3.335025
6	7.929985	6.625
7	7.589633	7.258583

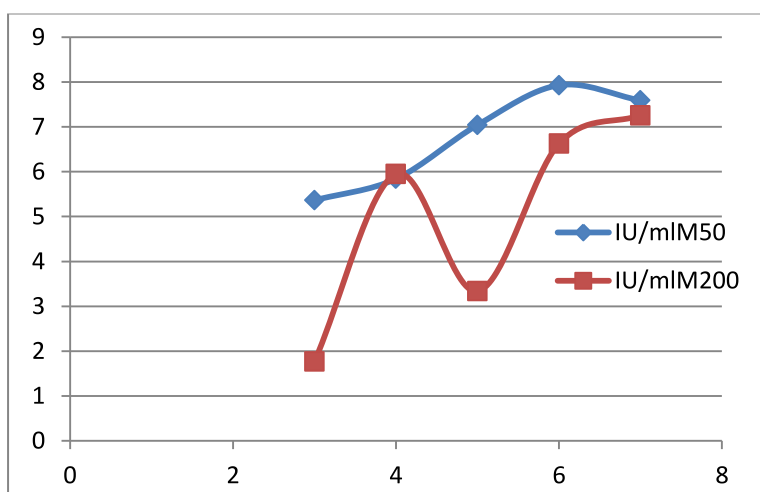


Figure-1.2-CMCassay of MM50ml & MM200ml

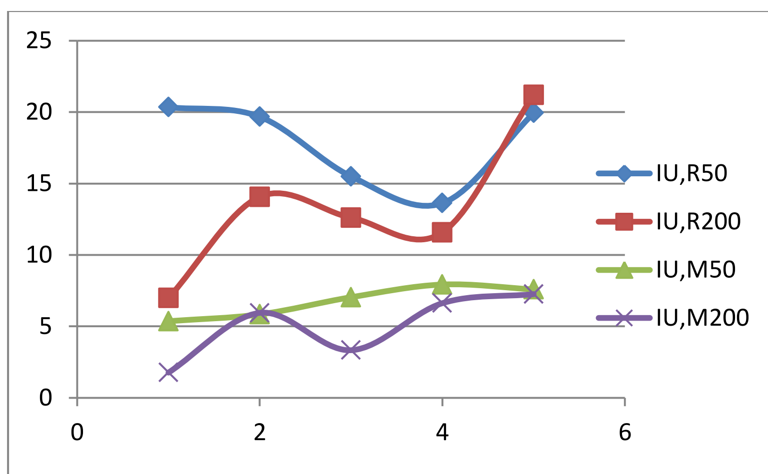


Figure-1.3-Comparison of RCM50,RCM200,MM50,MM200 CMC Assay

1.3.Result of Xylanase assay of RCM 50ml & RCM 200ml:

DAY	IU/ML,MM 50ml	IU/ML,MM 200ml
3	6.512531	5.215734
4	6.610704	6.018355
5	6.047337	6.171534
6	6.730897	6.024709
7	5.840633	5.441585

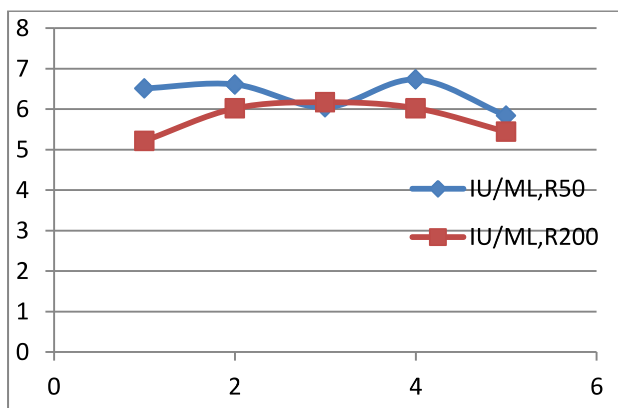


Figure-1.4-Xylanae assay of RCM50ml&RCM200ml

1.4.Xylanase assay of MM 50ml & MM 200ml:

DAY	IU/ML,MM 50ml	IU/ML,MM 200ml
3	6.338464	6.122621
4	6.058217	6.582157
5	6.334112	6.973807
6	7.038212	5.958999

7	5.815207	5.725749

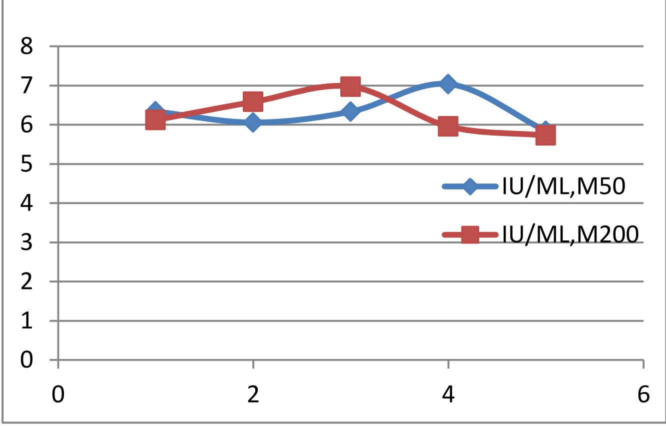


Figure-1.5-Xylanase assay of MM50ml & MM 200ml

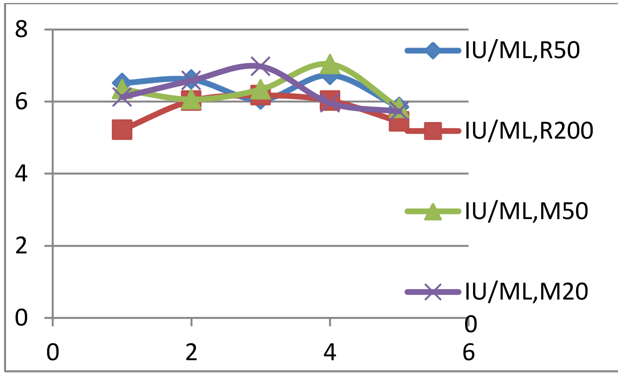


Figure-1.6-Comparison of xylanase assay among RCM50,RCM200,MM50&MM200

1.5.FPU assay of RCM 50ml,RCM 200ml,MM 50ml & MM 200ml:

DAY	FPU,RCM50	FPU,RCM200	FPU,MM50	FPU,MM200
5	2.533313	0.747016	0.99455	0.699868
6	3.547609	1.012494	1.566711	1.072915

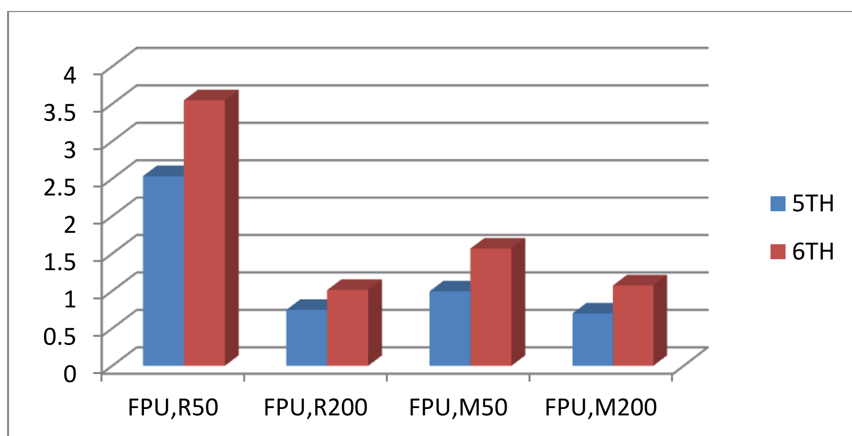


Figure-1.7-Comparison of FPU assay among rcm50,rcm200,mm50&mm200

2.DETERMINATION OF PACKED CELL VOLUME & DRY WEIGHT OF PENICILLIUM FUNICULOSUM SPORE AT DIFFERENT SPRE CONCENTRATION IN 1:10,SURFACE YO VOLUME RATIO:

From figure -1.8,1.9,1.10 it had been concluded that 10^6 spore/ml was shown better Packed Cell Volume & dry weight than 10^5spore/ml and 10^7spore/ml of *Penicillium funiculosum* wild type stain.

2.1.For 10^5 spore/ml

TIME	PCV in %	Dry weight(gm/L)
24 hours	5.708333	0.3625
48 hours	20.41667	1.5

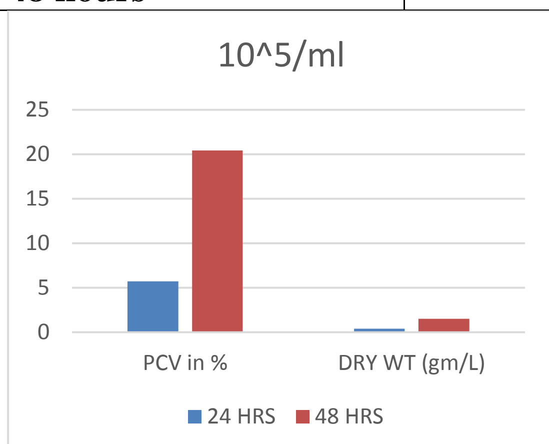


Figure-1.8-PCV & Dry weight of 10^5/ml sprore concentration of P.funiculosum wild type

2.2.For 10^6 spore/ml

TIME	PCV in %	Dry weight(gm/ml)
24 hours	8.708333	1.625
48 hours	24.58333	2.166667

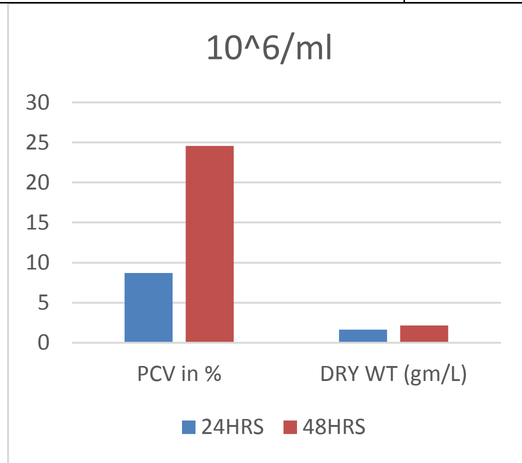


Figure-1.9-PCV & Dry weight of 10 ^{6}/ml sprore concentration of P.funiculosum wild type

2.3.For 10^7 spore/ml

TIME	PCV in %	Dry weight(gm/ml)
24 hours	12.04167	1.708333
48 hours	20.41667	1.916667

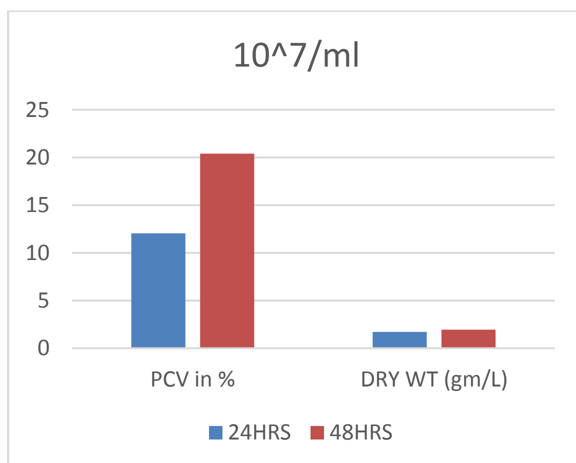


Figure-1.10-PCV & Dry weight of 10^7/ml sprore concentration of P.funiculosum wild type

3.RESULT OF CELLULASE ACTIVITY OF PENICILLIUM FUNICULOSUM,WILD TYPE STRAIN IN FERMENTOR:

For the industrial production purpose we mainly optised the cellulase production in 21 litre stirred tank reactor in batch operation using modified RCM media from *Penicillium funiculosum* .And it has been seen that endogluconase production from *Penicillium funiculosum* wild type was optimum on 9th day and it increase gradually from 3rd day th 9th day in 21 litre fermenter(figure-1.11).In figure-1.12 it had been seen that both PCV & FPU was optimum on 9th day in 21litra fermentor from *Penicillium funiculosum* wild type.

Endogluconase production from *Penicillium funiculosum* cre A mutant strain was increasing from 2nd day and reached optimum on 6th day and then decreased in 21 itra fermentor(figure-1.13)

In figure-1.14 it had been seen that FPU was increasing from *Penicillium funiculosum* cre A mutant stain from 1st day & reached optimum on 5th day and then decreased.

In figure-1.15, it had been seen lesser production of endogluconase in 21 litre stirred tank reactor from *Penicillium funiculosum* wild type stain than cre A mutant strain.Also enhanced production of FPU had been seen for cre A mutant than wild type strain of *Penicillium funiculosum.*

3.1.CMC Assay of Cellulase from Penicillium funiculosum,wild type in fermentor:

DAY	IU/ML,
3	0.6361
4	5.838
5	1.3519
6	0.3831
7	8.417
8	9.462
9	16.02

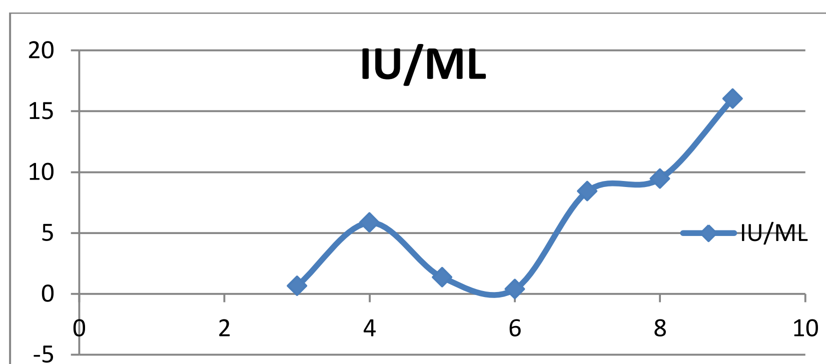


Figure-1.11-CMC assay result of cellulase from *P.funiculosum* wild type in fermentor

3.2.Result of FPU & Packed Cell Volume in fermentor of Penicillium funiculosum wild type:

DAY	FPU	PCV
3	0.05082	20%
4	0.07	25%
5	0.03	28%
6	0.041	16%
7	0.656	30%
8	0.6825	**44%**
9	1.177	**50%**

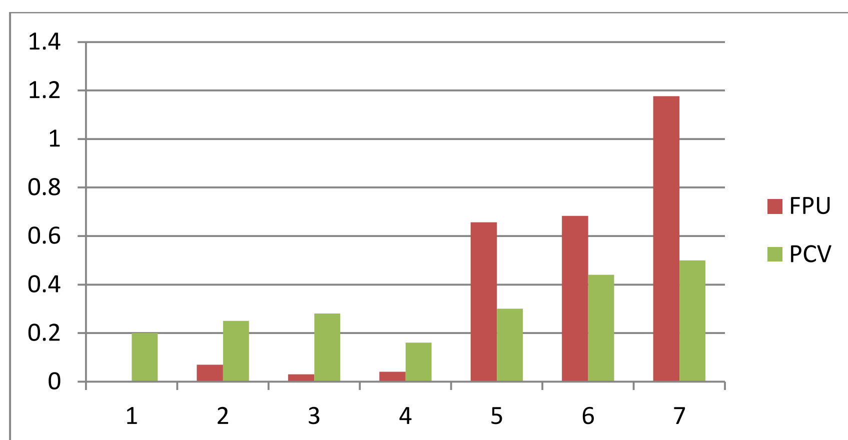


Figure-1.12- FPU Assay & PCV in fermenter of *P.funiculosum* wild type

4.RESULT OF CELLULASE ACTIVITY OF *PENICILLIUM FUNICULOSUM*,creA MUTANT STRAIN IN FERMENTOR:

4.1.CMC Assay of Cellulase from Penicillium funiculosum,wild type in fermentor:

DAY	IU/ML
2	24.87
3	26.02
4	32.057
5	25.73
6	35.29
7	34.711
8	27.0915
9	25.527

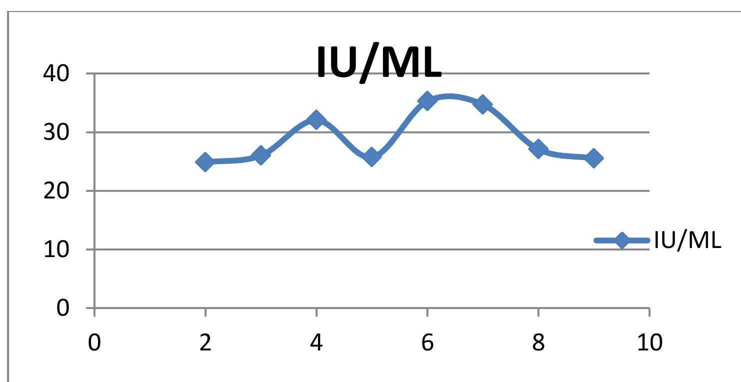


Figure-1.13- CMC assay result of cellulase from *P.funiculosum* cre A mutant in fermenter

4.2.Result of FPU in fermentor of Penicillium funiculosum wild type:

DAY	FPU
1	0.048964
2	0.544951
3	1.391847
4	1.239111
5	1.820539
6	1.503737
7	1.563536
8	0.30546

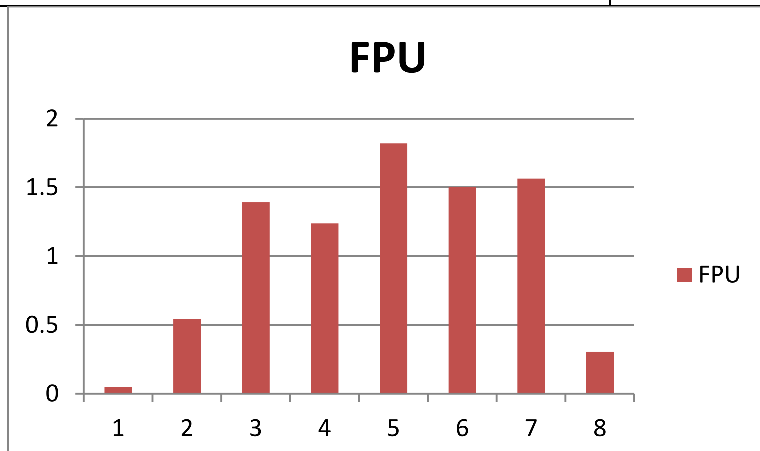


Figure-1.14-FPU assay of *P.funiculosum* creA mutant in fermentor

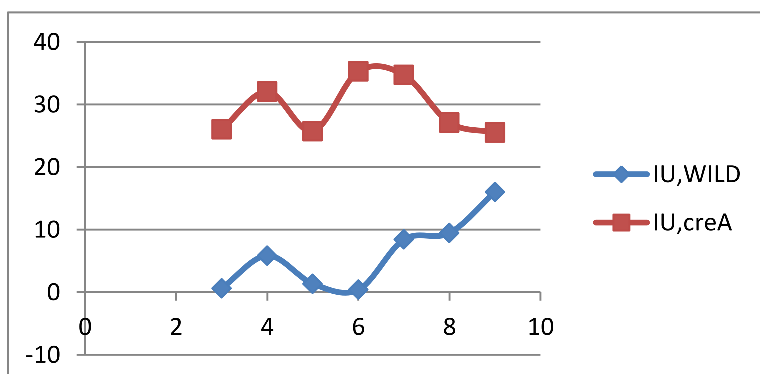


Figure-1.15-Comparison of CMC assay of wildtype & creA mutant stain of *P.funiculosum*

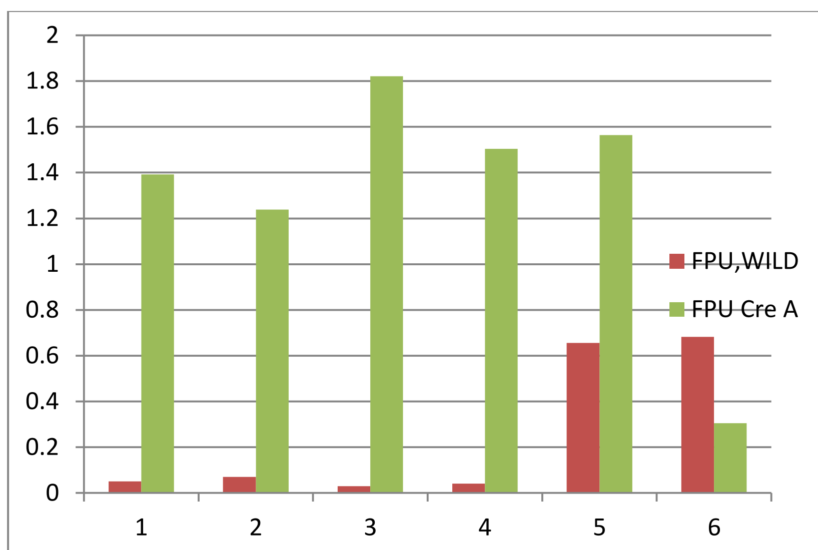


Figure-1.16-Comparison of FPU assay of *Penicillium funiculosum* wild type & cre A mutant stain in bioreactor

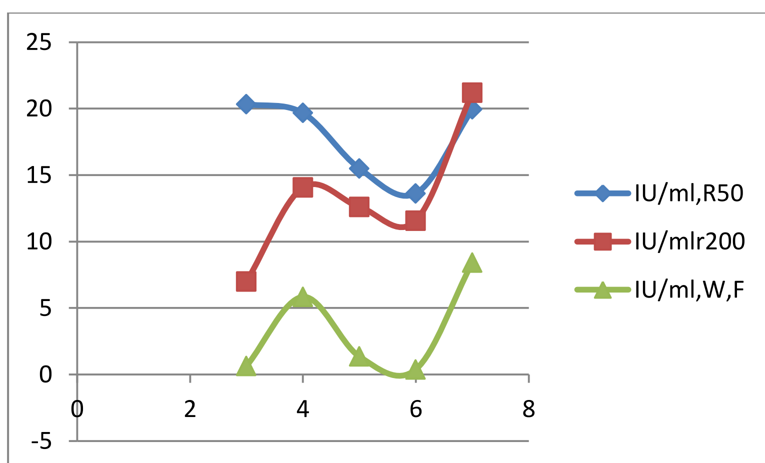


Figure-1.17-Comparison of FPU assay of *Penicillium funiculosum* wildtype in RCM50ml,RCM200ml &21litre fermenter

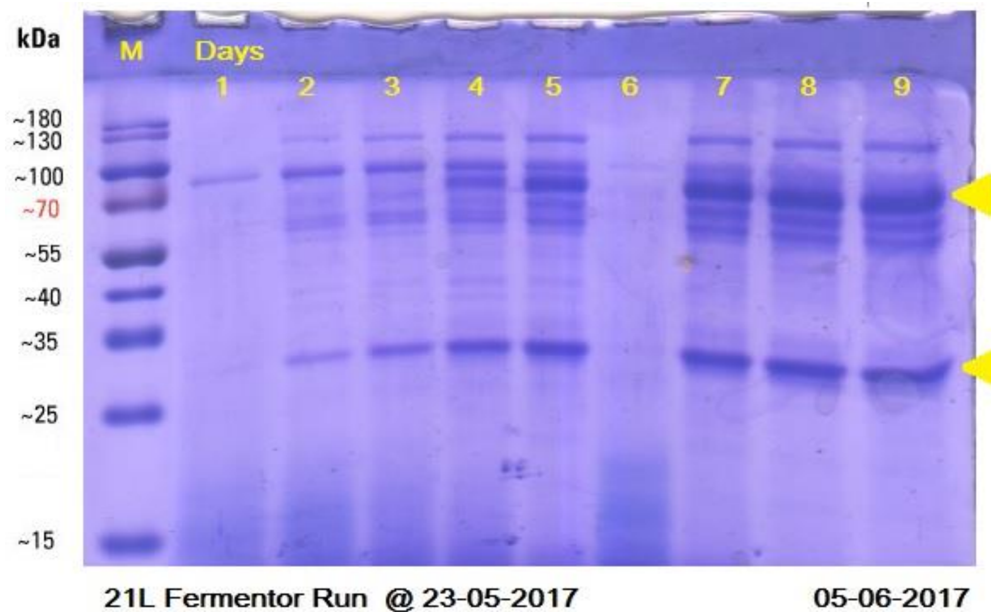


Figure-1.18-SDS PAGE of 1st to 9th day sample of 21 litre bioreactor,*P.funiculosum* wild type

In figure -1.18 wide bands were seen on 7th,8th & 9th day.It had been seen that endogluconase production was decreased in 21 litre bioreactor than small scale. In volumetric effect on cellulase production analysis in two media it has been seen RCM media with rich peptone concentration give better result than MM media.

And as mentioned in the review of literature that **Bischofberger et al.**observed that incubation of groundwater led to significantly more growth (about 2 log units) in small bottles (100 ml) than in big ones (10 liters).The same phenomenon has been seen in cellulase production ,in our project that CMC IU/ml,FPU & Xylanase was greater in small volume 50 ml volume than 200 ml volume.

Conclusion

From above experiment it is concluded that for volumetric effect & media optimization experiment, small volume 50 ml volume give greater FPU,CMC(IU/ML) & Xylanase (IU/ML) than 200 ml volume for both RCM & MM media.And FPU,CMC(IU/ML) & Xylanase (IU/ML) were greater in RCM media rather than MM media.In maximam cases of CMC,Xylanase & FPU assay enzyme production was optimum on 5th & 6th day.

And 10^6 spore/ml gave better Packed Cell Volume after 48 hours

In 21 litre fermentor FPU & endogluconase production was leeser from *Penicillium funiculosum* wild type may be due to shear inhibition
But for *Penicillium funiculosum* cre A mutant strain in 21 litre, fermentor shown enhanced FPU & CMC(IU/ml) than wild type as in cre A mutant stain the cell division rate is enhanced by 5 times that wild type so it shown enhanced productivity & give optimum FPU & CMC,IU/ml unit on 5th and 6th day,where wild type give it on 9th day.

Future Perspective:

Thus if the cellulase production is optimise with media & strain improvement procedure we can reach the goal to blend the ethanol of 10% in India as bioethanol production will enhanced.This improve the economy of India & others country.
Of the advanced biofuel processes, majority of the global R&D work is focused on the production of ethanol from lignocellulosic biomass. Various factors that affect successful emergence of biofuels industry include the extent of sustainable biomass supply, the kinds of products that can be produced, the nature of the conversion processes employed, the ability to efficiently extract the energy content of biomass, and the economics of large-scale plants to be built. Second-generation biofuel could be produced using a variety of non-food crops. The surplus biomass available in India viz. the stalks of wheat, rice, corn, pine needles, bamboo, sugarcane tops, cotton & chilli plant stalks, food-processing wastes etc. have been identified as potential non-food, non-feed bio-resources for their conversion into biofuels.

Dentifying this crucial need, Technology Information, Forecasting & Assessment Council (TIFAC), New Delhi and the National Institute for Interdisciplinary Studies on Science & Technology (NIIST), Thiruvananthapuram have conducted an in-depth nationwide study on current generation, consumption and availability patterns of surplus biomass from select agro & forest residues for their effective exploitation.

REFERENCES

Arai T, Matsuoka S, Cho H-Y, Yukawa H, Inui M, Wong S-L, Doi RH (2007). Synthesis of *Clostridium cellulovorans* minicellulosomes by intercellular complementation . Proc. Natl Acad. Sci. U.S.A. 104:1456–1460.

Arnold FH (2001). Combinatorial and computational chal-lenges for biocatalyst design. Nature 409:253–257.

Arnold FH, Wintrode PL, Miyazaki K, Gershenson A (2001).
How enzymes adapt: Lessons from directed evolution.
Trends Biochem. Sci. 26:100–106.

Baker JO, McCarley JR, Lovett R, Yu CH, Adney WS, Rignall TR, Vinzant TB, Decker SR, Sakon J, Himmel ME (2005). Catalytically enhanced endocellulase Cel5A .

Agis, M., A. Granda, and J. R. Dolan. 2007. A cautionary note: examples of possible microbial community dynamics in dilution grazing experiments. J. Exp. Mar. Biol. Ecol. **341:**176-183.

Amy, P. S., and H. D. Hiatt. 1989. Survival and detection of bacteria in an aquatic environment. Appl. Environ. Microbiol. **55:**788-793.

Bischofberger, T., S. K. Cha, R. Schmitt, B. König, and W. Schmidt-Lorenz. 1990. The bacterial flora of non-carbonated, natural mineral water from the springs to reservoir and glass and plastic bottles. Int. J. Food Microbiol. **11:**51-71.

Butterfield, C. T. 1933. Observations on changes in numbers of bacteria in polluted water. J. Sew. Works **5:**600-622.

Ferguson, R. L., E. N. Buckley, and A. V. Palumbo. 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47:**49-55.

Hammes, F. A., and T. Egli. 2005. New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum. Environ. Sci. Technol. **39:**3289-3294.

Hammes, F., M. Berney, Y. Wang, M. Vital, O. Köster, and T. Egli. 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Res. **42:**269-277.

Heukelekian, H., and A. Heller. 1940. Relation between food concentration and surface for bacterial growth. J. Bacteriol. **40:**547-558.

Jürgens, K., J. M. Gasol, and D. Vaqué. 2000. Bacteria-fla

H. Jørgensen and L. Olsson, “Production of cellulases by Penicillium brasilianum IBT 20888: effect of substrate on hydrolytic performance,” Enzyme and Microbial Technology, vol. 38, no. 3-4, pp. 381–390, 2006. View at Publisher · View at Google Scholar · View at Scopus

J. Sheehan, “The road to bioethanol: a strategic perspective of the U.S. Department of Energy’s National Ethanol Program,” in Glycosyl Hydrolases for Biomass
Conversion, J. O. Baker and J. N. Saddler, Eds., pp. 2–25, American Chemical Society, Washington, Wash, USA, 2001. View at Google Scholar
Z. Barta, K. Kovacs, K. Reczey, and G. Zacchi, “Process design and economics of on-site cellulase production on various carbon sources in a softwood-based ethanol plant,” Enzyme Research, vol. 2010, Article ID 734182, 8 pages, 2010. View at Publisher · View at Google Scholar · View at Scopus
H. Jørgensen, T. Eriksson, J. Börjesson, F. Tjerneld, and L. Olsson, “Purification and characterization of five cellulases and one xylanase from Penicillium brasilianum IBT 20888,” Enzyme and Microbial Technology, vol. 32, no. 7, pp. 851–861, 2003. View at Publisher · View at Google Scholar · View at Scopus
M. Linde, M. Galbe, G. Zacchi**Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration**