

**OPTIMISATION OF XYLANASE ENZYME
PRODUCTION IN *ASPERGILLUS* Spp.**

A PROJECT REPORT

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ABSTRACT

The aim of this project was to optimize the operating parameters for the production of Xylanase using a wild strain of *Aspergillus* (species unknown) by submerged batch fermentation using semi-automatic equipment. This was the first attempt to use a fungal culture to produce enzymes at Spic Biotech Ltd. (a major producer of industrial enzymes in India), which has so far been using only bacterial cultures. This project was a continuation of the laboratory – scale work done by Spic Biotech’s R&D division and constituted the first factory trials for this process. As part of the project, several trial batches were conducted in a 200 liter fermenter with various agitation and aeration rates. By monitoring these batches for pH, viscosity, Packed Mycelial Volume and Enzyme activity, and studying their trends, we arrived at the optimum operating parameters. Also, the design specifications of a 200 liter fermenter were quantified and scaled up for a 1500 liter fermenter and a 9000 liter fermenter.

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LIST OF SYMBOLS

P_o - Power absorbed from the agitator when no gas was sparged in g cm/sec

P_g - Gassed power in g cm/sec

g_c - Newton's Law conversion factor in cm/sec²

N - Impeller rotational speed in sec⁻¹

D_i - Impeller diameter in cm

ρ - Density of the fluid in g/cm³

N_p - Power Number (Dimensionless)

N_a - Aeration Number (Dimensionless)

Q - Volumetric gas flow rate in cm³/sec

OTR - Oxygen Transfer Rate

$K_L a$ - Volumetric oxygen transfer coefficient

$(C^* - C_L)$ - Driving force for mass transfer

α, δ - Constants relating to the number of impellers

N_i - Number of impellers

V_L - Volume of liquid inside the fermenter in l

P_g/V_L - Gassed power per unit volume in hp/1000 l

V_s - Superficial gas velocity in cm/min

X - Cell concentration in g/l

N - Cell concentration in cells/l

t - Time

μ - Specific growth rate in hr⁻¹ (mass)

μ_n - Specific growth rate (number)

t_d - Doubling time

R - Pellet radius

M - Biomass

ρ_M - Density of biomass

W_{Xylose} - Weight of Xylose

DF - Dilution Factor (Dimensionless)

V_{enzyme} - Volume of enzyme solution in ml

PMV - Packed Mycelial Volume in %

V - Volume of fermenter in m^3

H - Height of fermenter in m

D_t - Diameter of fermenter in m

D_i - Diameter of impeller in m

J - Width of baffle in m

W - Width of impeller in m

L - Length of impeller in m

E - Distance from the lowest impeller to the base of the fermenter in m

H_{actual} - Actual height of fermenter in m

T - Thickness of fermenter wall in mm

P - Pressure inside fermenter in N/mm^2

F – Permissible stress in N/mm

J' - Joint efficiency (Dimensionless)

C - Corrosion allowance in mm

1.0 INTRODUCTION

1.1 ENZYMES

Enzymes are an important class of globular proteins of biological origin that act as biochemical catalysts. The most distinguishing property of an enzyme in its catalytic action is its specificity and selectivity – each enzyme catalyses only a specific reaction involving a specific substrate. Hence their great value to chemists and engineers. Another major characteristic of enzymes is their sensitivity to the conditions in which they operate – they are functional only within a specific range of pH, temperature and presence of inhibitors, cofactors etc. A very useful property of enzymes as catalysts is that they are generally required in very small quantities.

To get an idea of the potency of enzymes, consider the following facts:

- It would take our digestive tract about 50 years to digest a single meal without enzymes.
- The hydrolysis of a peptide which would require very drastic conditions such as prolonged heating with a base, in the absence of enzymes, occurs under rather moderate conditions (as prevalent in a living system) in their presence.
- Enzymes can speed up an uncatalysed reaction by as much as 10 million times.

As in the case of biochemical reactions, we observe a great variety in enzymes too – so far about 3700 enzymes have been classified.

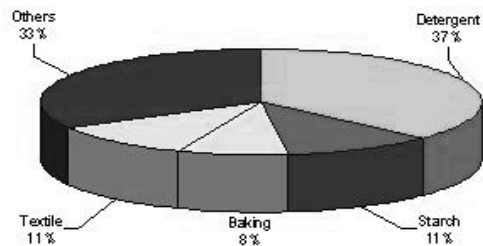
1.1.1 COMMON TYPES OF ENZYMES

ENZYME	REACTION CATALYZED	
HYDROLASES	General term for enzymes that catalyze a hydrolytic cleavage reaction.	<p>Enzyme names typically end in “-ase” with the exception of some enzymes such as pepsin, trypsin, thrombin, lysozyme and so on which were discovered and named before the convention became generally accepted at the end of the nineteenth century. The common name of the enzyme usually indicates the substrate and the nature of the reaction catalyzed. For example, citrate synthase catalyzes the synthesis of citrate by the addition of acetyl CoA to oxaloacetate.</p>
NUCLEASES	Break down nucleic acids by hydrolyzing bonds between nucleotides.	
PROTEASES	Break down proteins by hydrolyzing bonds between amino acids.	
SYNTHASES	General term for enzymes that synthesize molecules in anabolic reactions by condensing two smaller molecules together.	
ISOMERASES	Catalyze the rearrangement of bonds within a single molecule.	
POLYMERASES	Catalyze polymerization reactions such as the synthesis of DNA and RNA.	
KINASES	Catalyze the addition of phosphate groups to molecules. Protein kinases are an important group of kinases that attach phosphate groups to proteins.	
PHOSPHATASES	Catalyze the hydrolytic removal of a phosphate group from a molecule/	
OXIDO-REDUCTASES	General name for enzymes that catalyze reactions in which one molecule is oxidized and the other is reduced. They are also called <i>oxidases</i> , <i>reductases</i> and <i>dehydrogenases</i> .	
ATPASES	Hydrolyze ATP. Many proteins with a wide range of roles have an energy-harnessing ATPase activity as part of their function, for example, motor proteins such as <i>myosin</i> and membrane transport proteins such as the <i>sodium-potassium pump</i> .	

1.1.2 SCOPE OF ENZYME INDUSTRY

Global Enzyme User Industries

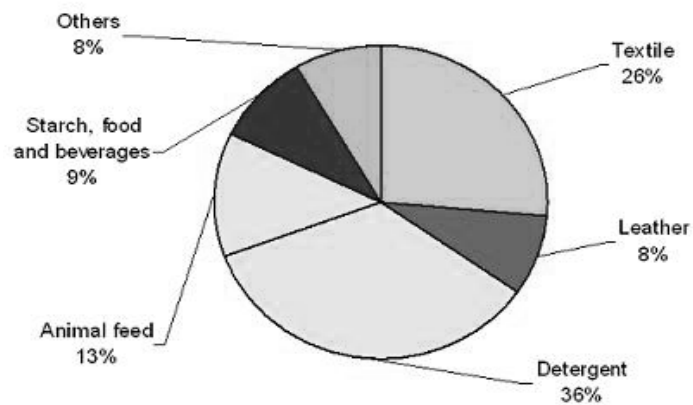
US \$ 1800 M (2000)



Source : Business Communication Company Inc.

Industrial Enzymes The Indian Scenario

Rs. 160 Cr



Source : Business Communication Company Inc.

GLOBAL ENZYME MARKETS BY APPLICATION SECTORS THROUGH 2009

	(\$ Million)				
	2002	2003	2004	2009	%AAGR 2004-09
Technical Enzymes	978.2	1009.2	1040.0	1222.0	3.3
Food Enzymes	701.0	720.0	740.0	863.0	3.1
Animal Feed Enzymes	210.8	215.6	220.0	267.0	3.9
Total	1890.0	1945.0	2000.0	2352.0	3.3

1.2 XYLANASE

It is a hydrolase that catalyses the hydrolysis of complex sugars (primarily xylan and certain related compounds) to simpler sugars (the primary product being xylose).

- Systematic Name: *1,4-β-D-xylanohydrolase*
- Recommended Name: *endo-1,4-β-xylanase*
- Type: Hydrolase
- Substrate: Xylan
- Product: Xylose
- Sources:
 - Bacterial: *Bacillus polymyxa*, *Cryptococcus albidus* etc.
 - Fungal: *Aspergillus spp.* (*nidulans*, *ochraceus*, *fumigatus*), *Trichoderma spp.* etc.
- pH Range: Acidic (3.5 – 7.0)
- Optimum pH: 5.3
- Temperature Range: 35 – 60 °C
- Optimum Temperature: 55 °C

The product being produced at SPIC Biotech Ltd. is acidic xylanase and the source of the enzyme a fungus belonging to the *Aspergillus* genus.

1.2.1 APPLICATIONS OF XYLANASE

a) **Kraft Pulp Bleaching:**

Xylanase is used in paper industry to improve the strength of cellulose fibers in bleached Kraft Pulp (from bamboo, eucalyptus etc.).

It improves pulp fibrillation and water retention, reduces beating times in virgin pulps, restores bonding, increases freeness in recycled fibers and selectively removes xylan from dissolving pulps.

b) **De-inking of Newsprint:**

Cellulase blended with xylanase in specific ratios is used for deinking newsprint. At optimum ratio (50-50), brightness of deinked pulp is higher, ink removal ratio is lower and breaking length, bursting index and tearing index are higher than for chemically deinked pulp. This process also gives a higher yield than chemical deinking.

c) **In Poultry Industry:**

Xylanase is added to poultry feed material such as rice/wheat bran along with various other enzymes to improve absorption of nutrients by the birds and to reduce the quantity and nutrient concentration of the birds' droppings.

d) In Wine Production:

It is used to break down xylans, pectin and hemicellulose present in fruits into simpler molecules such as xylose and glucose. By breaking down cell walls, it helps extract more juice from the fruit.

e) In Baking:

Xylanase breaks down glycosidic linkages in arabinoxylans (in endo or exo fashion), producing smaller fragments. This improves the handling properties of dough, the over-spring and the bread volume.

f) In Forage Digestions:

Xylanase along with other fibrolytic enzymes is sprayed onto forages in specific total mixed ratios and fed to lactating dairy cows to enhance FCM and milk production.

g) Agricultural Waste Degradation

h) Yielding Cellulose from Rayon

1.3 FERMENTATION

1.3.1 BASICS

Fermentation, chemical changes in organic substances produced by the action of enzymes. This general definition includes virtually all chemical reactions of physiological importance, and scientists today often restrict the term to the action of specific enzymes, called ferments, produced by minute organisms such as molds, bacteria, and yeasts. For example, lactase, a ferment produced by bacteria usually found in milk, causes the milk to sour by changing lactose (milk sugar) into lactic acid. Fermentation is a process in which an agent causes an organic substance to break down into simpler substances through the action of catalysis.

Fermenter, a system consisting of a few pieces of equipment which provide controlled environmental conditions for the growth of microbes (and/or the production of specific metabolites) in liquid culture whilst preventing entry and growth of contaminating microbes from the outside environment.

Fermentation process – a basic outline

- Formulation of media required to support the growth of the organism being used in the process. (Seed medium and Production medium)
- Sterilization of media, fermenters and ancillary equipment.

- Production of pure, active culture of the organism being used, in a sufficient quantity to inoculate the fermenter vessel (Sub-culturing and Seed fermentation).
- Growth of the organism in the production fermenter under optimum conditions for the formation of the desired product.
- Extraction of the product from the broth and its purification.
- Disposal of effluents produced during the process.

Fermenter – basic components and their uses

The main components of a fermenter are as follows:

- base components including drive motor, heaters, pumps, gas control etc;
- vessel and accessories (agitator impeller, aerator etc);
- peripheral equipment such as reagent vessels;
- instrumentation and sensors.

The above components combine to perform the following functions:

- provide operation free from contamination;
- maintain a specific temperature;
- provide adequate mixing and aeration;
- control the pH of the culture;
- allow monitoring and/or control of dissolved oxygen;
- allow feeding of nutrient solutions and reagents;
- provide access points for inoculation and sampling;
- use fittings and geometry relevant to scale-up;

- minimize liquid loss from the vessel;
- facilitate the growth of a wide range of organisms.

1.3.2 FERMENTATION PROCESSES: NATURE AND SCOPE

Commercially important fermentation processes may be broadly classified under four heads:

- **Those that produce microbial cells as the product**
- **Those that produce microbial enzymes as the product**
- **Those that produce microbial metabolites as the product**
- **Those that modify a compound that is added to the fermenter – transformation process.**

The process under study falls under the second category, the enzyme xylanase being the desired product.

Technically, fermentation processes may be classified as

- **Solid Substrate Fermentation**
- **Submerged Fermentation**

Solid Substrate Fermentation

Microorganisms are cultivated on a solid substrate with a high content of nutrients and a large surface area, such as wheat germ, wheat bran, rice bran and cereal meal, with the addition of mineral salts. The low water content of the medium considerably lowers the threat of bacterial infections. Also, the

enzyme solution obtained is more concentrated in comparison to that obtained in a submerged fermentation process.

The major downside of this method of production is that it is not possible to feed ingredients to the medium during cultivation. As a result, the changes in pH and carbohydrate concentration can not be controlled during the process and are predetermined by the composition of the medium and inoculum.

Submerged Fermentation

In this case, microorganisms are cultivated in a liquid medium in which contains the required nutrients in the required concentrations. Nutrients are generally supplied in the form of cheap and readily available materials such as rice bran and wheat bran (sources of carbohydrates) or in pure and concentrated forms such as bacteriological peptone (source of proteins) which are either dissolved or suspended in water to form the growth medium. The medium is fed into a CSTR (Continuous Stirred Tank Reactor) where it is incubated at a specified temperature and pressure with constant aeration and agitation, until sufficient concentration of product is reached.

The advantage of this process is the ease with which various parameters can be monitored (by periodic sampling of broth) and controlled if necessary by the addition of further nutrients or reagents. Surface-active agents are often used to prevent foaming and to cause increased excretion of extra-cellular enzymes.

Based on the mode of operation, fermentation processes may be classified into 3 types:

- **Batch processes**
- **Fed-batch processes**
- **Continuous processes**

Based on the type of organisms used, fermentation processes are classified as follows:

- **Those that use bacteria/yeast/fungi**
- **Those that use plant cells**
- **Those that use a mammalian cell culture**
- **Those that use algae**

1.3.3 ENZYME FERMENTATIONS

The main reason for the attractiveness of microorganisms as potential enzyme sources is the ease with which enzyme levels may be increased by environmental and genetic manipulations. Thousand-fold increase have been recorded for catabolic enzymes and biosynthetic enzymes have been increased several hundred-fold. Of course, the higher the specific activity, the simpler will be the job of enzyme isolation. Other reasons for using microbial cells as sources of enzymes are:

1. Enzyme fermentations are economical on a large scale because of short fermentation cycles and inexpensive media.

2. Screening procedures are simple and thousands of cultures can be examined in a reasonably short time.
3. Different species produce somewhat different enzymes catalyzing the same reaction, allowing flexibility with respect to the desired operating conditions in the reactor.

Factors Important to Enzyme Fermentations

- **Strain Selection**

- In devising enzyme fermentation, it is important to begin with the most active strain available.

- **Improving Fermentation**

- Once a good strain is obtained, fermentation parameters must be optimized to maximize growth and enzyme production. Of importance here are temperature, pH, and oxygen transfer. Also important is the nutrition of the microorganism, especially with respect to sources of carbon, nitrogen, phosphorous, sulfur, and mineral salts. Often, especially, with extra-cellular enzymes, the addition of surfactants is important.

- **Recognizing Growth Cycle Peak**

- If the fermentation is conducted as a batch culture, the stage of the growth cycle at which enzyme content is highest must be ascertained. In many cases enzymes rapidly disappear after reaching their peak activity.

- **Other factors to be considered**
 - Induction
 - Feedback Repression
 - Catabolite Repression
 - Mutants Resistant to Repression
 - Gene Dosage

1.4 FACTORS AFFECTING FERMENTATION

According to Levenspiel, the most important factors to be considered in designing a reactor are

- i. the selection of the best type of reactor for the reaction, and
- ii. determination of the best operating conditions.

The scale of operation and kinetics of the given reaction are usually determined beforehand. There is considerable freedom of choice with regard to other features. A batch process or any of various types of continuous processes may be adopted and within limits, any initial concentration of reactants, operating temperature and pH can be used.

The major factors affecting a fermentation process are,

- i. Nature of the biochemical reaction – homogenous / heterogenous
- ii. Rate processes (rate controlling step)
- iii. Operational considerations
- iv. Local conditions within a fermenter
 - a. Residence time distributions

- b. Concentration distributions
- c. Temperature distributions

The most important factor to be considered during the operation of a fermenter is the provision of adequate mixing of its contents. The main objectives of mixing in fermentation are to disperse the air bubbles, to suspend the microorganisms (or animal and plant tissues), and to enhance heat and mass transfer in the medium.

Since most nutrients are highly soluble in water, very little mixing is required during fermentation just to mix the medium as microorganisms consume nutrients. However, dissolved oxygen in the medium is an exception because its solubility in a fermentation medium is very low, while its demand for the growth of aerobic microorganisms is high.

For example, when the oxygen is provided from air, the typical maximum concentration of oxygen in aqueous solution is on the order of 6 to 8 mg /L. Oxygen requirement of cells is, although it can vary widely depending on microbes, on the order of 1g/L h. Even though a fermentation medium is fully saturated with oxygen, the dissolved oxygen will be consumed in less than 1 minute by the organisms if not replenished continuously. Adequate oxygen supply to cells is often critical in aerobic fermentation. Even temporary depletion of oxygen can damage cells irreversibly. Therefore, gaseous oxygen / air must be supplied continuously to meet the requirements for high oxygen needs of microorganisms, and oxygen transfer can be a major rate limiting step for cell growth and metabolism.

a. Laboratory scale operation:

The gentle mixing and surface aeration provided by the rotary or reciprocating action of a laboratory shaker apparatus is adequate to cultivate microorganisms in flasks or test tubes.

b. Large scale operation:

For bench-, pilot- and production-scale fermenters, mixing is provided by mechanical agitation with or without aeration. A radial flow impeller with six flat blades mounted on a disk (aka flat-blade-disk turbine or Rushton turbine) is the most widely used arrangement.

Radial-flow impellers produce flow radially from the turbine blades towards the side of the vessel, where the flow splits into two directions: one part goes upward along the side, back to the center along the liquid surface, and down to the impeller region along the agitating shaft; and the other goes downward along the side and bottom, then back to the impeller region. On the other hand, the axial flow impellers generate flow downward to the tank bottom, then up the side and back down the center to the impeller region. Therefore, the flat-blade disk turbine has the advantage of limiting the short-circuiting of gas along the drive shaft by forcing, introduced from below, along the path into the discharge jet.

Purpose of Agitation

Agitation promotes oxygen transfer in four ways.

- a. By dispersing air in the culture fluid in the form of fine bubbles culminating in the increase of gas liquid interfacial area available for oxygen transfer.
- b. It delays the escape of air bubbles from the fluid, i.e. it increases the gas hold-up or bubble retention time.
- c. It prevents coalescence of air bubbles.
- d. By creating turbulence in the culture fluid, it decreases the thickness of the liquid film at the gas-liquid interface.

1.4.1 POWER REQUIREMENTS IN AGITATED FERMENTORS

Power absorption depends on two factors,

- i. Rheological properties of the broth – whether it is Newtonian or Non-Newtonian.
- ii. Whether the system is gassed or non-gassed.

Most fermentation media exhibit Newtonian behavior until they are inoculated with micro-organisms.

Power absorption is generally characterized by the Power Number (N_p), which is a ratio of the external force exerted to the inertial force imparted to the fluid.

$$N_p = \frac{P_o g_c}{N^3 D_i^5 \rho}$$

where P_o is the external power from the agitator in g cm/sec, g_c is the Newton's Law conversion factor in cm/sec², N is the impeller rotational speed in sec⁻¹, D_i is the impeller diameter in cm, and ρ is the density of the fluid in g/cm³.

In multiple impeller systems, maximum power absorption and mixing occur when the impellers are properly spaced – between one to two diameters apart. If the impellers are thus properly spaced, the power absorbed by the fermentation broth will be directly proportional to the number of impellers.

In a gassed system showing Newtonian behavior, the two important parameters related to power absorption are Aeration Number (N_a) and Gassed Power. When air is sparged into a liquid system previously maintained at a certain rate of agitation, there will be a reduction in power absorption.

N_a is a ratio of superficial gas velocity to the impeller tip velocity and is given by

$$N_a = \frac{Q/D_i^2}{ND_i} \text{ (Dimensionless)}$$

where N_a is the dimensionless aeration number, Q is the volumetric gas flow rate in cm³/sec, D_i is the impeller diameter in cm, and N is the impeller speed in sec⁻¹.

Aeration number has been correlated as an independent variable to the ratio of the gassed power P_g to the power absorbed when no gas was sparged P_o .

Correlations have also been developed for the gassed power P_g in terms of the ungassed power requirement (P_o), the impeller speed (N), the impeller diameter (D_i) and a constant that is a function of geometry.

The equations developed for Newtonian broths hold good for Non-Newtonian broths with some modifications.

1.4.2 OXYGEN TRANSFER IN FERMENTATION SYSTEMS

Oxygen Transfer Rate (OTR) is given by the following equation typical of any mass transfer operation.

$$\text{OTR} = K_L a (C^* - C_L)$$

The mass transfer coefficient $K_L a$ may be regarded as the reciprocal of the overall resistance to the transfer of oxygen from the gaseous phase to the liquid phase. The expression $(C^* - C_L)$ quantifies the driving force for mass transfer. Neither the gas-liquid interfacial area nor the mass transfer coefficient can be readily measured in a sparged agitated fermenter. Therefore, these two terms are combined into one single parameter $K_L a$, the volumetric oxygen transfer coefficient.

When oxygen is transferred from an air bubble to a solid particle in a well mixed liquid system, application of Film Theory of Interfacial Mass Transfer suggests that three transport pathways are possible:

- Transfer of oxygen from the bulk gas phase across the gas film to the liquid interface.

- Transfer of oxygen from the interface across the liquid film to the bulk liquid.
- Transfer of oxygen from the bulk liquid across the second liquid film to the solid phase that is mixed in the bulk liquid.

Thus an oxygen molecule has to overcome all these resistances to reach the microbial cell.

Mass Transfer in Large-Scale Fermenters

In laboratory equipment the operating variables such as gas flow rate, impeller speed, impeller diameter, and others, can all be easily varied to accommodate changes in oxygen transfer rate. Furthermore, there is little economic consequence if a laboratory fermenter is over-designed or under-designed with respect to the agitation horse power requirement or the compressor volumetric delivery. For large-scale operations, one does not have this flexibility.

Unfortunately, useful data for large-scale fermenter operations are extremely sparse in the literature.

With the larger volume fermentors, multiple impellers are employed. When the mass transfer coefficients for such cases are examined with respect to the power input, there is always a discontinuity when the number of impellers is increased. Therefore, it is necessary to incorporate the effect of multiple impellers in order to correlate the mass transfer behavior.

It has been found that the power per unit volume (P_g/V_L) and the superficial gas velocity V_s were useful independent parameters for correlating the quantitative behavior of the oxygen mass transfer coefficients.

Correlation of Mass Transfer Coefficient

The mass transfer coefficient $k_L a$ can be correlated by the following equation which resembles the general equation except for the use of the additional parameter N_i , the number of impellers:

$$k_L a = (\alpha + \delta N_i) (P_g/V_L)^{0.77} V_s^{0.67}$$

where $k_L a$ is the overall mass transfer coefficient in mM / l-h r/ atm, α , δ are the constants relating to the number, N_i is the number of impellers, P_g/V_L is the gassed power per unit volume in hp/1000 l and V_s is the superficial gas velocity in cm/min .

The exponent of power per unit volume is believed to generally decrease with increasing scale of operation. Also, the volumetric scale of operation does not seem to affect the exponent on the superficial gas velocity. This is encouraging since, in large fermenters, the volumetric air flow rate or the superficial gas velocity is much more easily varied than the agitator power input.

1.5 FERMENTATION PROCESSES USING BACTERIA / YEAST / FUNGI

Either a natural isolate of the culture is made or an uncontaminated master culture is revived from a freeze-dried state or from liquid nitrogen storage. The starting cultures are grown in Petri dishes on agar containing the required nutrients and incubated at an optimum temperature, humidity and gaseous atmosphere. If necessary, shake-flask cultures are prepared in an incubator shaker to produce larger volume of culture for analysis or for use as an inoculum for a fermenter. Flasks are typically filled to 20-25% to provide a large area for gas exchange and are 25ml to 5l in total capacity. An inoculum is prepared either from the shake-flask culture or by suspending colonies from growth on solid media in a nutrient/buffer solution. This is aseptically transferred to a fermenter vessel. The inoculum is typically 5-10 % of the volume of medium in the fermenter. A laboratory scale fermenter can be used to provide large volumes (typically 1-5l of culture). Growth is normally as batch fermentation with the following parameters measured and controlled as necessary:

Temperature, typically 20-40 °C (4-90 °C at the extremes)

Speed, typically 150-1500 rpm (20-2000 rpm at the extremes)

pH, typically 4 - 8 (2 - 12 at the extremes)

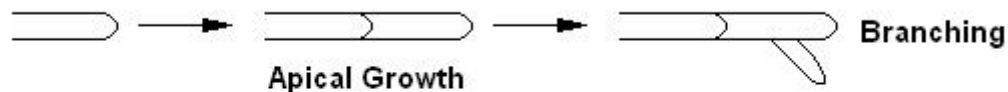
Dissolved oxygen, typically 40 - 80% (0 - 100% at the extremes)

Samples are periodically taken from the fermenter to monitor various parameters. On completion of the batch, the whole broth is centrifuged to separate the whole cells from the supernatant liquid. Alternatively, a membrane or hollow-fiber system can also be used for initial separation.

1.6 FUNGAL GROWTH KINETICS

1.6.1 GROWTH OF MYCELIA

Fungal mycelia characteristically grow by chain elongation and branching as illustrated below.



MYCELIAL GROWTH

Growth proceeds from the tip of the mycelium (apical growth) by forming septa between the cells. Depending on the physiochemical environment, the mycelium may be long and diffuse, short and highly branched, or a mixture of the two. When grown on a surface, the mycelia may become intertwined and form thick mats. While in submerged culture the mycelia may exist as diffuse mycelia or may form pellets with diameters ranging from 0.1 – 10 mm. The growth of a fungal pellet is illustrated in the figure below, by the nutrient concentration.

It is very difficult to follow growth with respect to cell number in molds because the cells are not easily separated. For this reason increases in mass

are used to monitor growth. Actinomyces and Streptomyces are classes of organisms closely related to bacteria that are extremely important industrially. Both classes also grow as mycelial organisms.

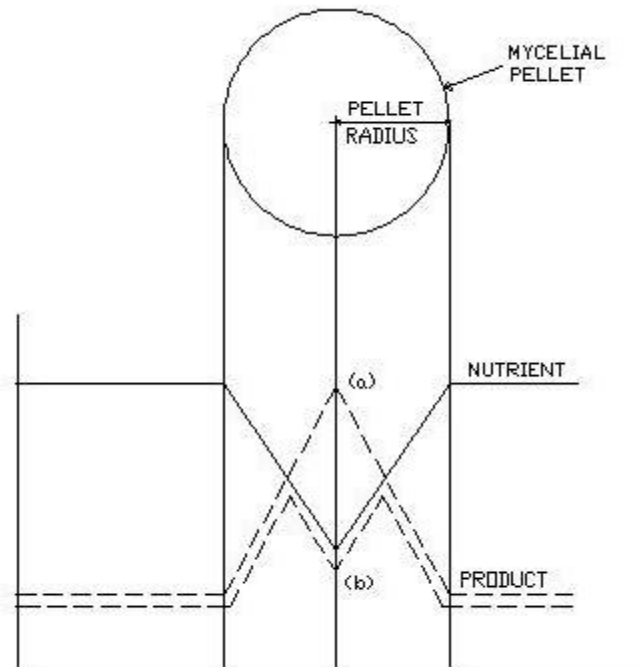


Figure: Nutrient and product concentration profiles in a mycelial pellet; if product is synthesized in the center of the pellet, the profile will look like (a); if the cells in the center are dead or nutrient starved, the product profile will look like (b).

1.6.2 MICROBIAL GROWTH KINETICS

Microbial growth is usually characterized by the time required to double cell mass or cell number. Mass doubling time may differ from cell doubling time because cell mass can increase without increase in cell number. However, if in a given environment the interval between cell mass or number doublings

is constant with time, the organism is growing at an exponential rate. Under such conditions, growth is described by the following equations

$$\frac{dX}{dt} = \mu X \quad (\text{e 1.1})$$

or

$$\frac{dN}{dt} = \mu_n N \quad (\text{e 1.2})$$

where X = cell concentration in g/l

N = cell concentration in cells/l

t = time

μ = specific growth rate in hr^{-1} (mass)

μ_n = specific growth rate (number)

Thus equation e 1.1 describes the increase in cell mass with time and equation e 1.2 describes the increase in cell number with time. Under most circumstances, growth is measured by an increase in mass, so μ will be used. The value μX is the volumetric growth rate (volumetric productivity) in g/l-hr.

Integration of equation e 1.1 gives,

$$\int_{X_1}^{X_2} \frac{dX}{X} = \int_{t_1}^{t_2} \mu dt \quad (\text{e 1.3})$$

If the specific growth rate is constant, Equation e 1.3 yields

$$\ln \frac{X_2}{X_1} = \mu \Delta t \quad (\text{e 1.4})$$

Equation e 1.4 may be solved the case in which $\Delta t = t_d$, that is, the time required for $X_2 = 2X_1$ (doubling time), then;

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \quad (\text{e 1.5})$$

From equation e 1.4, it is that the specific growth rate is obtained from the slope of a plot of $\ln X$ versus time.

1.6.3 MICROBIAL GROWTH KINETICS IN A BATCH PROCESS

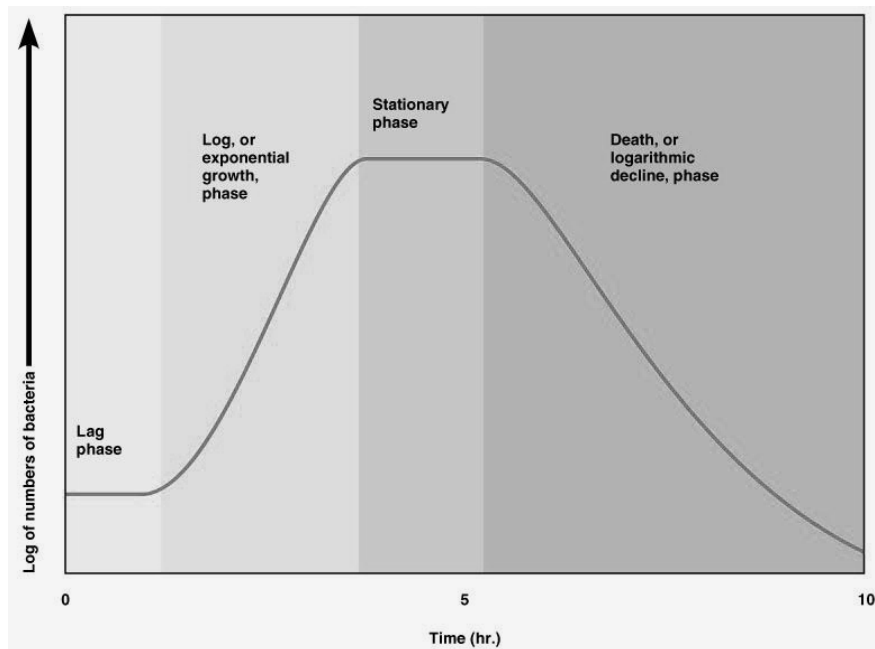
A batch culture is a typical closed system containing a limited amount of nutrients, added initially. The inoculated culture, as it grows, passes through various stages. The growth kinetics is described by various equations, all derived from the fundamental *Monod Equation*.

- **Lag Phase** – This is an initial phase just after inoculation when no growth appears to take place, i.e. the change in cell number is zero or negligible. However, cells may grow in size during this period. This period may be explained as the time taken by the organism to adapt to the prevalent conditions.
- **Accelerated Growth Phase** – Growth begins at the end of the lag phase, the division rate increases gradually and reaches a maximum in the exponential phase.

- **Log / Exponential Phase** – This is a phase where the cells grow at an increasing rate due to progressive doubling of cell number. A microbial culture undergoing balanced growth is analogous to a first order autocatalytic reaction.

However, growth is accompanied by the consumption of nutrients, and excretion of auto toxic bio-products by the organism, events that lead to a decline in growth rate after a certain length of time. Due to such effects, growth starts to decrease after a certain amount of time, finally coming to a stop altogether.

- **Decelerated Growth Phase** – Towards the end of the log phase, growth starts to slow down, the division rate decreases gradually and reaches zero in the stationary phase.
- **Stationary Phase** – Growth of microbes becomes limited due to exhaustion of nutrients or accumulation of toxic metabolic products or both.
- **Death Phase** – In this stage, the population of the organisms starts to decline. Cell death occurs due to ageing effects produced in part by the depletion of energy reserves/sources and/or accumulation of toxic metabolic products. And similar to growth, death also follows an exponential function.



1.6.4 GROWTH OF FILAMENTOUS ORGANISMS

In fungi and other such filamentous organisms, mass and morphology of a fungal pellet or pulp varies as growth proceeds. Experimental studies of submerged batch cultures indicate that mass increases at a slower than exponential rate with proportionality to $(\text{time})^3$ providing a reasonable approximation of the data. Such a growth pattern can be rationalized from observation of one- and two-dimensional fungal cultures. In the first instance, the rate of increase in the colony length is constant while the radius of the fungal colony increases at a constant rate in surface culture (2-dimensional).

Extrapolating to a spherical pellet growing in submerged culture, let us assume that

$$\frac{dR}{dt} = k_g = \text{const} \quad (\text{e 2.1})$$

where R denotes the pellet radius. Since the biomass M is given by,

$$M = \rho_M \frac{4}{3} \Pi R^3 \quad (\text{e 2.2})$$

we have from equations e 2.1 and e 2.2,

$$\frac{dM}{dt} = \rho_M 4\Pi R^2 \frac{dR}{dt} = k_g 4\Pi R^2 \rho_M \quad (\text{e 2.3})$$

2.3)

Eliminating R from (e 2.3), using (e 2.2), leaves

$$\frac{dM}{dt} = \gamma M^{\left(\frac{2}{3}\right)} \quad (\text{e 2.4})$$

where

$$\gamma = k_g (36\Pi\rho_M)^{1/3} \quad (\text{e 2.5})$$

Integrating equation (e 2.4) with an initial biomass of M_o , yields

$$M = \left(M_o^{1/3} + \frac{\gamma t}{3} \right)^3 \quad (\text{e 2.6})$$

Since M_o is usually quite small relative to M, equation (e 2.6) gives the cubic dependence of M on t mentioned above.

A complete analysis of filamentous organisms' growth should also consider the kinetics of pellet formation. This occurs due to agglomeration of spores and subsequent growth or by outgrowth from an individual spore. Research to date has shown that many properties of the organism and its growth environment interact to influence pellet formation. Because the mechanisms

are complicated and not sufficiently well understood or documented, general kinetic models for pellet formation have not yet been developed.

When considering growth of existing pellets, the model outlined above must be viewed as a crude approximation of reality. Pellet size, morphology, and internal structure, all of which are expected to influence pellet kinetics, are determined by interactions among agitation intensity, pellet concentration, organism properties and medium composition. Overall kinetics of pellets often depends upon diffusion – chemical reaction interactions.

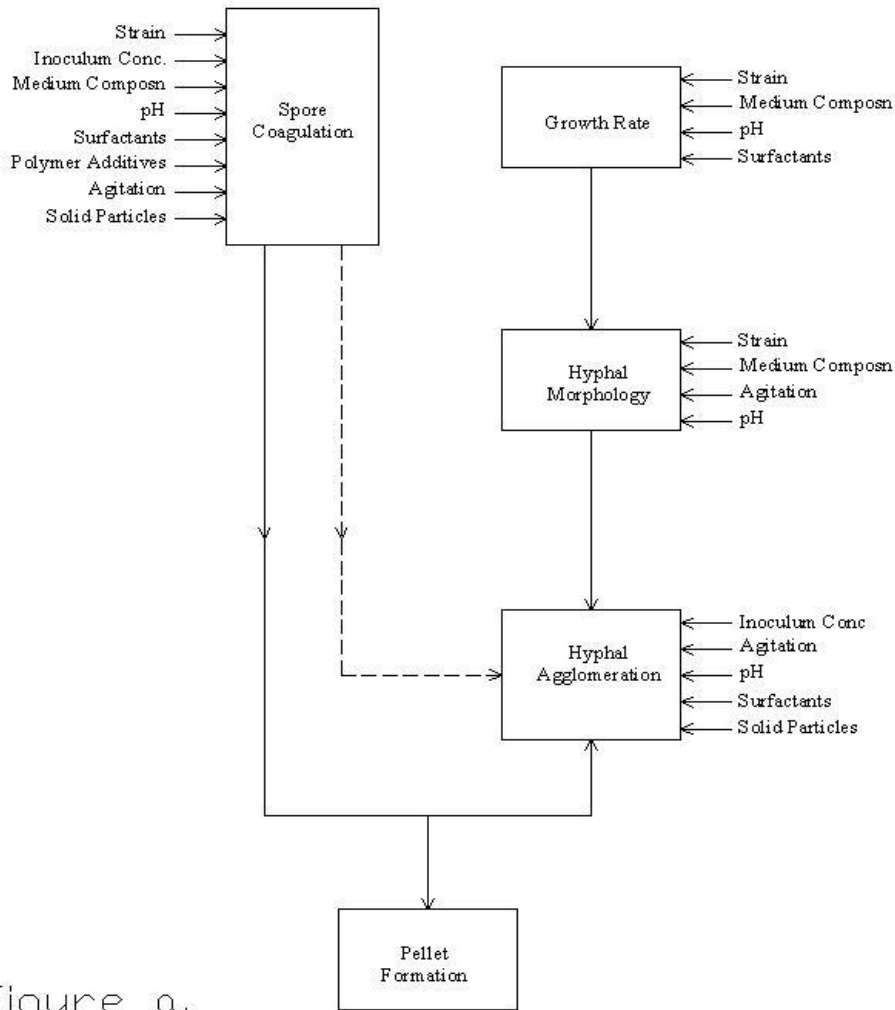


Figure a.

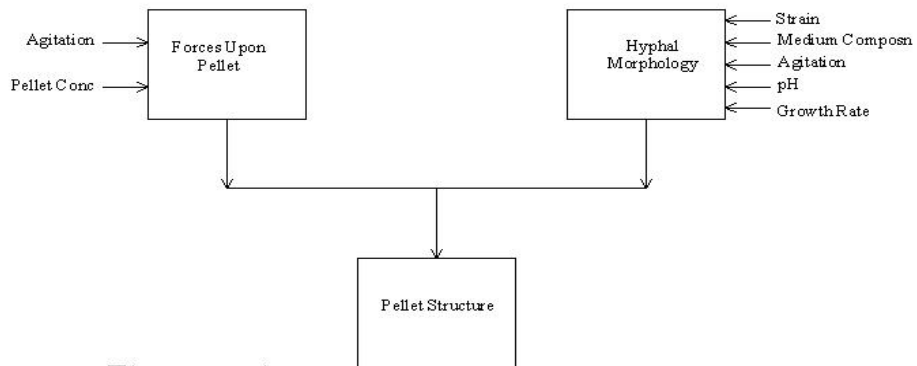


Figure b.

Figure: Summary of factors which interact to determine (a) pellet formation and (b) pellet structure during cultivation of mycelial organisms.

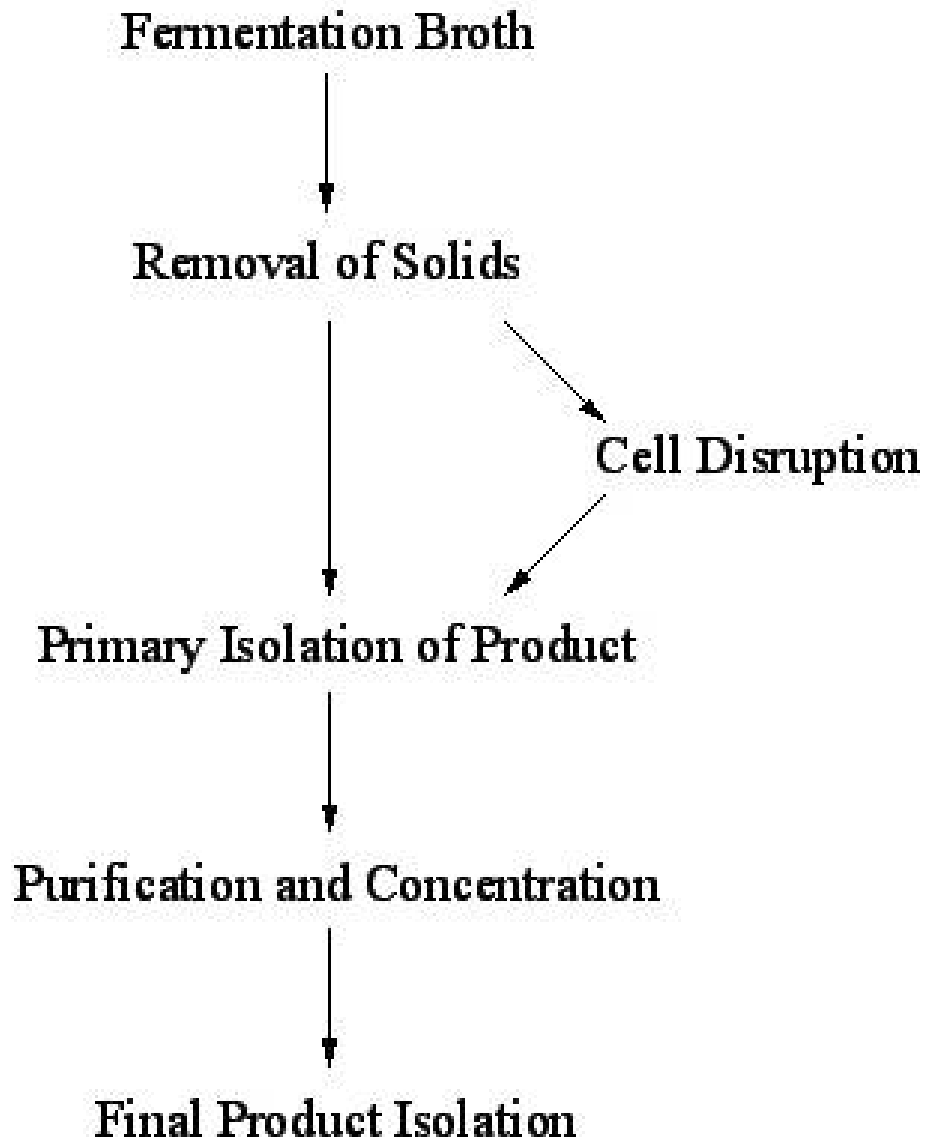
1.7 DOWNSTREAM PROCESSING

Downstream processing (DSP) refers to the recovery of the product to the desired purity and concentration. This is often one of the most critical steps in a process, technically as well as economically. It is very important to convert the product to a stable final product that is easy to store and use and has a sufficiently high concentration of the active compounds. Products in fermentation processes are found in complex mixtures and in very low concentrations. The products, being bio-molecules are extremely sensitive and there are constraints with regard to the pH, temperature, salt or solvent compositions. This is especially true of enzymes as their activity depends upon the structural integrity of the macro molecule as well as its secondary and tertiary structures.

The choice of recovery process is based on the following criteria:

1. The intra-cellular or extra-cellular location of the product.
2. The concentration of the product in the fermentation broth.
3. The physical and chemical properties of the desired product.
4. The intended use of the product.
5. The minimal acceptable standard of purity.
6. The magnitude of bio-hazard of the product or broth.
7. The impurities in the fermenter broth.
8. The marketable price of the product.

Stages in product recovery from a harvested fermentation broth



Range of processes applicable to DSP of fermentation products:

- Foam Separation
- Precipitation
- Filtration
 - Batch Filtration
 - Cross Flow Filtration
- Centrifugation
- Cell Disruption
 - Physico-mechanical Methods
 - Liquid Shear
 - Solid Shear
 - Agitation with abrasives
 - Freeze-thawing
 - Ultrasonication
 - Chemical Methods
 - Detergents
 - Osmotic Shock
 - Alkali treatment
 - Enzyme treatment
- Liquid-Liquid Extraction
- Two-Phase Aqueous Extraction
- Supercritical Fluid Extraction
- Chromatography
 - Adsorption Chromatography
 - Ion-exchange Chromatography

- Gel Permeation Chromatography
- Affinity Chromatography
- Reverse Phase Chromatography
- High Performance Liquid Chromatography
- Membrane Separation
 - Ultrafiltration
 - Reverse Osmosis
 - Liquid Membranes
- Drying
- Crystallization

Each type of product necessitates specific techniques in DSP, intracellular products being more difficult to recover. The product being produced in the process under study is extracellular acidic xylanase. Hence, the special techniques applied for the recovery of intracellular products is deemed to be beyond the purview of this discussion.

General steps for DSP of extra-cellular enzymes produced by submerged culture:

- Extraction
- Solid-liquid separation
- Concentration
- Precipitation
- Drying (optional)
- Milling (optional)
- Granulation (optional)

Product	Typical Process Sequence	Known Alternatives
Extracellular Enzymes	1. Flocculate slurry and cool.	1. Ultra-filter in presence of solids.
	2. Separate solids (rotary or press filter or centrifuge).	2. Recover enzyme concentrate directly from slurry by electrophoresis, selective adsorption, two-phase aqueous partition, direct precipitation, etc.
	3. Concentrate filtrate by ultrafiltration or vacuum evaporation.	3. Additional purification prior to concentration (example: heat treat to reduce nucleic acid content).
	4. Formulate and standardize concentrate for sale or precipitate solid by ethanol or ammonium sulphate, filter off and dry. Formulate solid for sale in attractive physical form. Recover ethanol by distillation.	4. Recover enzyme from concentrate as fixed product on appropriate support material.
	5. For greater purity products, purify concentrate by adsorption / chromatographic processes (ion exchange resins, gel filtration).	

- **Spray Drying**

A spray drier is most widely used for drying of biological materials when the starting material is in the form of a liquid or paste. The material to be dried does not come into contact with the heating surfaces. Instead, it is atomized into small droplets through for example a nozzle or by contact with a rotating disc. The droplets then fall into a spiral stream of hot gas at 150° to 250°. The high surface area to volume ratio of the droplets results in a rapid rate of evaporation and product size being directly related to droplet size produced by the atomizer. The evaporative cooling effect prevents the material from becoming overheated and damaged. The gas-flow rate must be carefully regulated so that the gas has the capacity to contain the required moisture content at the cool-air exhaust temperature (75° to 100°). In most processes the recovery of very small particles from the exit gas must be conducted using cyclone filters. This is especially important for containment of biologically active compounds. The jet spray drier is particularly suited to handling heat sensitive materials. Operating at a temperature of around 350°, residence times are approximately 0.01 seconds because of the very fine droplets produced in the atomizing nozzle.

Spray driers are the most economical available for handling large volumes, and it is only at feed rates below 6 kg min⁻¹ that drum driers become more economic.

- **Centrifugation**

Centrifugation is mainly used for solid liquid separation. The tubular, chamber and disk stack centrifuges rotate in a vertical position for continuous operation with respect to the liquid phase. Solid discharge may be discontinuous or continuous. Parameters of importance are the particle size, density and concentration of the particles and the liquid viscosity. Ultracentrifuges have by far the highest acceleration, leading to sequential sedimentation according to molecule mass, even for soluble components like proteins. Several options in the basic design of certain centrifuges are in the market. Certain equipment for biotechnological procedures can be operated under sterile conditions, with or without containment, under cooling and temperature control.

- **Chromatography**

It is a high resolution technique and therefore preferred if proteins of high purity are desired. It has been established for the production of highly purified insulin and plasma fractionation. Many DNA products of high volume need to be purified to homogeneity and chromatography is increasingly used. The popular chromatographic methods used are based on size and shape (gel filtration), charge (ion exchange), isoelectric point (chromato-focusing), surface free energy (hydrophobic separation) and bio-specific interaction (affinity and immuno adsorption). In the enzyme industry this process is only used when a highly purified product is necessary. Since the process is a costly one, a high selling price must be assured, for the process to be economically viable.

- **Membrane Separation**

- **Ultrafiltration**

Ultrafiltration can be described as a process in which solutes of high molecular weight are retained when the solvent and low molecular weight solutes are forced under hydraulic pressure through a membrane of a very fine pore size. It is therefore used for product concentration and purification. A range of membranes made from a variety of polymeric materials, with different molecular weight cut-offs are available which makes possible the separation of macromolecules such as proteins, enzymes, hormones and viruses. It is practical only to separate molecules whose molecular weights are a factor of ten different due to variability in pore size.

- **Reverse Osmosis**

Reverse osmosis is a separation process where the solvent molecules are forced by an applied pressure to flow through a semi-permeable membrane in the opposite direction to that dictated by osmotic forces, and hence is termed reverse osmosis. It is used for the concentration of smaller molecules than is possible by ultrafiltration. Concentration polarization is again a problem and must be controlled by increased turbulence at the membrane surface.

2.0 REVIEW OF LITERATURE

Kornelia Zetelaki and Karoly Vas have studied the influence of agitation and aeration on growth and on production of glucose oxidase of *Aspergillus niger* in submerged culture in *Biotechnology and Bioengineering* (1968). It was found that both growth rate and glucose oxidase production was higher at an agitation speed of 700 rpm than at 460 rpm. Further increase in speed of agitation resulted in neither a higher growth rate nor a higher glucose oxidase activity.

The production, purification and characterisation of xylanase from a hyperxylanolytic mutant of *Aspergillus ochraceus* was studied by S.R.Biswas, S.C.Jana, A.K.Mishra, and G.Nanda in *Biotechnology and Bioengineering* (1990).

J.J Smith and M.D. Lilly have discussed the effect of agitation on the morphology and penicillin production of *penicillium chrysogenum* in *Biotechnology and Bioengineering* (1997).

Xylanase production by *Aspergillus awamori* and the development of a medium and optimization of the fermentation parameters for the production of extracellular xylanase and β – xylosidase while maintaining low protease production was discussed by David.C.Smith, and Thomas.M.Wood in *Biotechnology and Bioengineering* (1991).

Y.Q.Cui, R.G.J.M. van der Lans, and K.C.A.M. Luyben have studied the effect of agitation intensities on fungal morphology in submerged fermentation in *Biotechnology and Bioengineering* (1997).

The effects of process parameters on heterologous protein production in *Aspergillus niger* fermentation were studied by Liping Wang, Daring Ridgway, Tingyue Gu, and Murray Moo-Young in *Journal of Chemical Technology and Biotechnology* (2003).

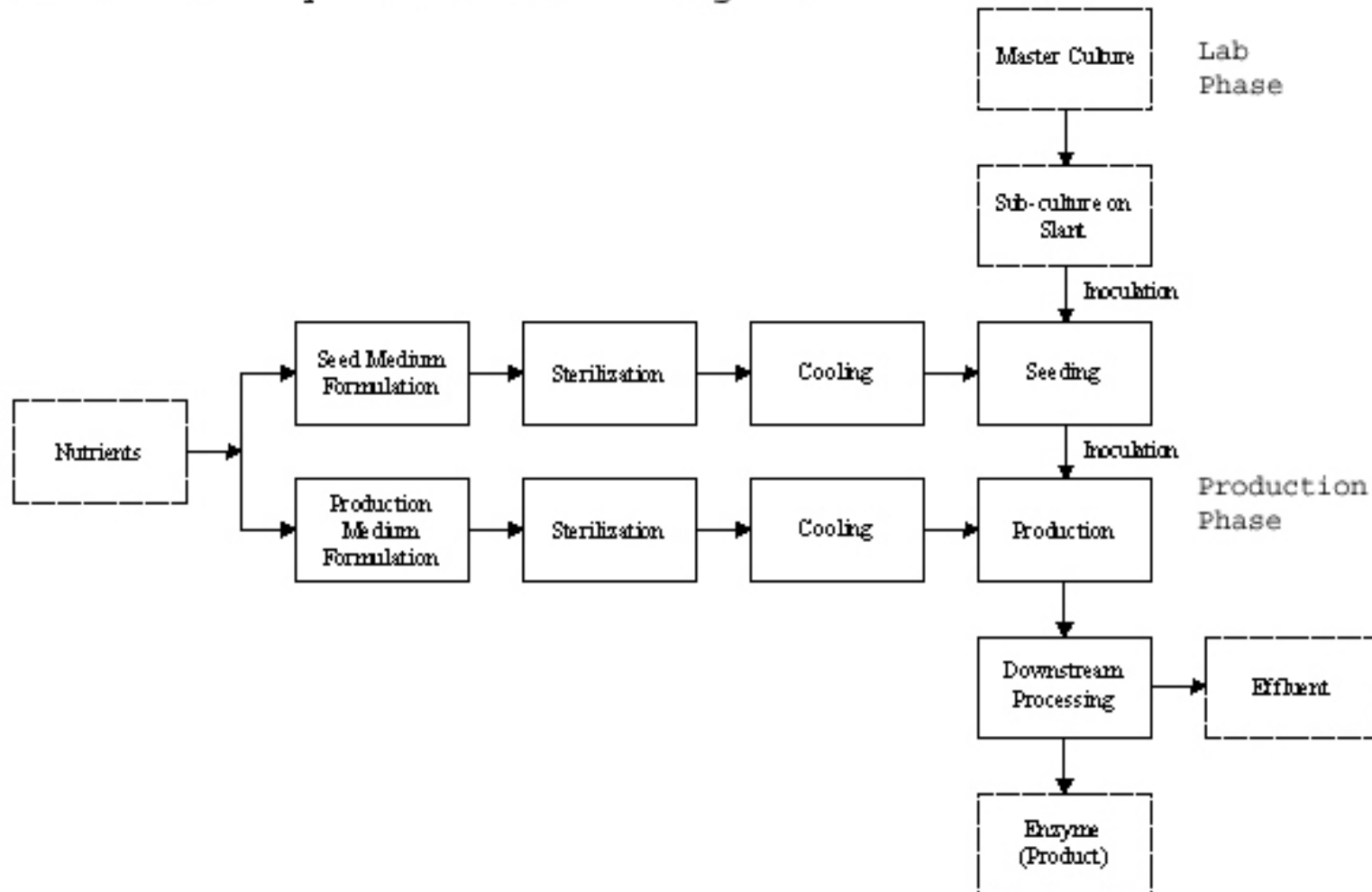
P.Jüsten, G.C.Paul, A.W.Nienow, and C.R.Thomas have studied the dependence of mycelial morphology on impeller type and agitation intensity in *Biotechnology and Bioengineering* (1996).

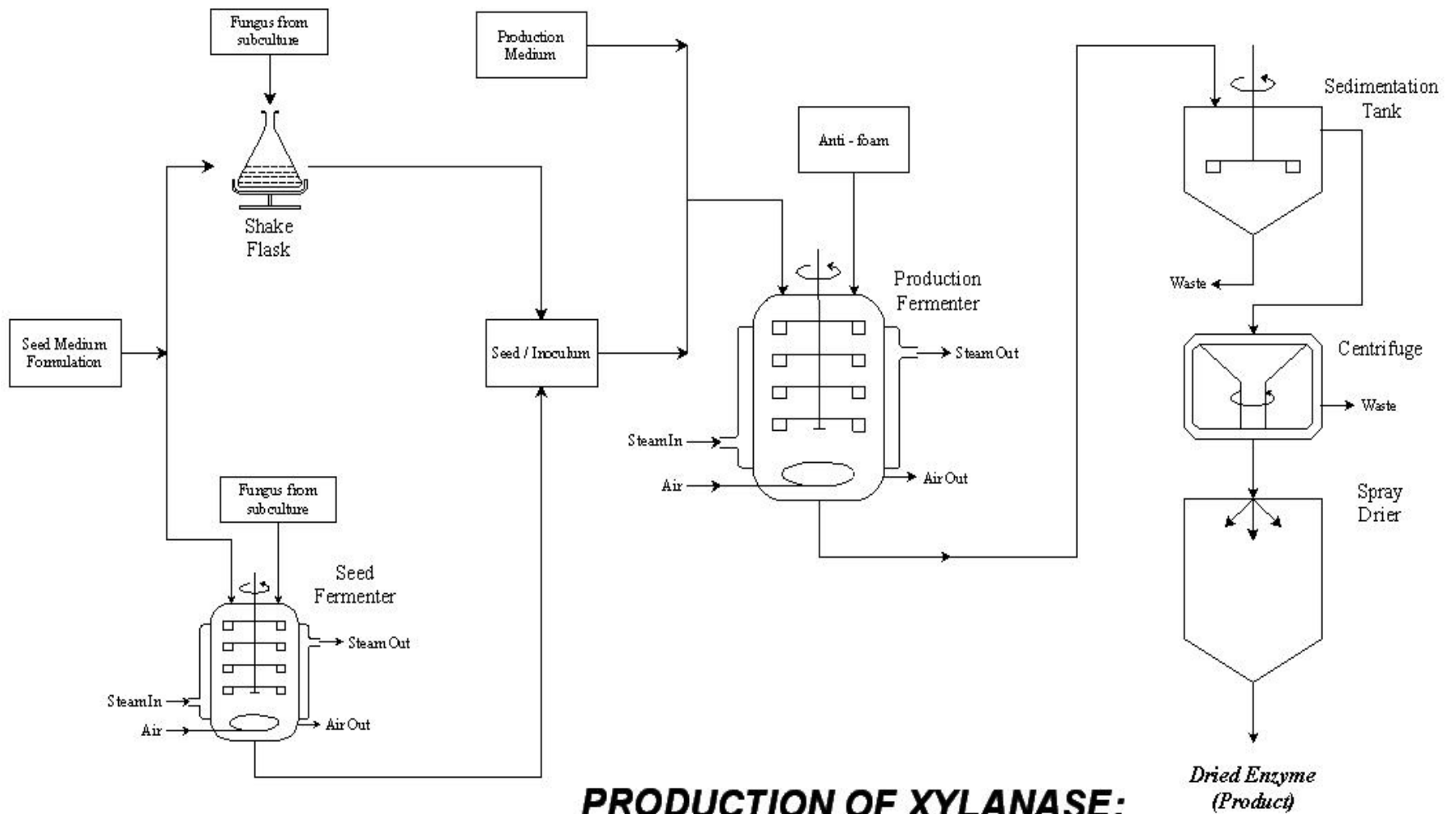
3.0 PROCESS DESCRIPTION

- A sub-culture of *Aspergillus* spp. is grown on a slant by streaking it with fungus from the original culture plate.
- The fungus from the sub-culture is inoculated into seed medium in a shake flask and placed on an orbital shaker for a period of about 24 hrs. The shaker helps aerate the seed broth and promotes fungal growth. The broth thus obtained is used to seed the fermenter.
- The production medium is prepared for about 35% of the capacity of the fermenter (200 liters), i.e. 70 liters.
- The medium is sterilized by heating it to 121 °C at a pressure of 1.2 Kgf/cm² and maintaining those conditions for 30 minutes.
- Heat is supplied by circulating steam through the fermenter jacket.
- Air is filtered by passing it through .22 micron polypropylene filter.
- Cold water is then circulated through the fermenter's jacket and the broth is cooled to about 30 °C.
- The production medium is inoculated with about 6 liters of seed.

- The pH and PMV of the seed before inoculation and the broth just after inoculation are measured.
- The process is begun by setting the values of aeration and agitation rates.
- The process is monitored continuously through periodic measurement of the following parameters:
 - Temperature
 - Operating Pressure
 - pH
 - PMV
 - Viscosity
 - Activity
- The samples collected are examined under a microscope to check for bacterial or other types of contamination.
- When the peak activity is reached, the batch is harvested after the addition of a biocide. The biocide kills the fungus and prevents any further bio-chemical reactions from taking place.

Production of Xylanase: Block Diagram





**PRODUCTION OF XYLANASE:
PROCESS FLOW SHEET**

PRODUCTION FERMENTERS AT SPIC BIOTECH LTD., PORUR



4.0 MATERIALS AND METHODS

4.1 ENZYME ASSAY

Definition of Unit of Activity

According to the International Union of Biochemistry, one enzyme international unit has been defined as the amount of enzyme that will catalyze the hydrolysis of 1 micromole of substrate per minute, at reaction conditions. In describing the activity of Xylanase, 1 IU corresponds to the amount of enzyme required to release 1 micromole of reducing sugar (xylose) in 1 minute at 40 °C and atmospheric pressure.

Principle

Xylanase catalyses the enzymatic hydrolysis of Xylan, (substrate) releasing Xylose. The reaction is arrested by the addition of 3,5 – dinitro salicylic acid (DNS). Also DNS forms a red colored complex with xylose. The amount of xylose released by the reaction is measured by measuring its absorbance at 540 nanometers using a spectrophotometer.

Instruments used

- Spectrophotometer (calibrated to measure optical density at 540 nanometers)
- Water bath (maintained at 60 °C)
- Water bath (maintained at 100 °C)
- Vortex Mixer
- Micropipettes

Chemicals used

- Enzyme sample (crude xylanase)
- Sodium acetate – acetic acid buffer (at a pH of 5.6)
- Xylose (substrate)
- 3,5 – dinitro salicylic acid (DNS) solution
- Distilled water

Procedure

- The enzyme sample to be tested was diluted to a suitable level of dilution (the dilution factor lying between 50 and 500)
- In a clean dry test tube, 1 ml of the diluted enzyme solution was taken. To it were added 1 ml of acetate buffer solution and 1 ml of substrate solution. The test tube was incubated at 60 °C in a water bath for 15 minutes.
- Also a blank was similarly prepared, but with the 1 ml of substrate solution replaced with 1 ml of distilled water. The blank was also incubated along with the sample tube.
- The reaction was arrested by adding 2 ml of DNS solution to each test tube.
- 500 µl of xylan solution was added to the blank test tube whereas an equal volume of distilled water was added to the sample test tube.
- The tubes were then boiled at 100 °C in a water bath for 10 minutes to allow the color to develop.

The test tubes were then cooled in an ice bath.

- The absorbance of the liquid in each tube was measured at 540 nm using a spectrophotometer.
- The OD value of the blank corresponds to the amount of xylose present in the enzyme sample and the substrate added. This is the amount of xylose that was already present and therefore was not released due to the reaction.
- Therefore the amount of xylose released due to the reaction is proportional to the difference between the OD values of the sample and blank.
- A standard graph is plotted between the OD of the solution and the weight of xylose. The equation of the standard graph was found experimentally to be :
$$OD = 0.2334 \times (\text{Weight of Xylose}) - 0.0652$$
- The weight of xylose corresponding to the $(OD_{\text{Sample}} - OD_{\text{Blank}})$ was determined from the graph.
- From this value the enzyme activity was calculated as follows:

$$\text{Enzyme Activity (in IU)} = \frac{W_{\text{Xylose}} \times DF}{V_{\text{enzyme}} \times t}$$

where W_{Xylose} -Weight of Xylose

DF - Dilution Factor

V_{enzyme} - Volume of enzyme solution in ml

t - Duration of the reaction in minutes)}

Result

The enzyme activity was determined as discussed above in each of the samples collected from the fermenter.

4.2 MEASUREMENT OF PACKED MYCELIAL VOLUME

Principle

In order to track the growth of fungi in the broth we measure the packed mycelial volume (PMV). The PMV is the fraction of the total broth volume occupied by the fungal mycelia. It is measured by separating the fungal mycelia from a known volume of broth sample by centrifugation and then measuring the volume of the supernatant remaining. The ratio of the difference between the volumes of the broth sample and the supernatant to the volume of the broth sample expressed as a percentage gives the PMV.

Instruments used

- Centrifuge

Procedure

- 1 ml of broth sample is taken in a centrifuge tube and centrifuged at a fixed rpm (say 10,000 rpm) for a fixed length of time (say 10 minutes).
- The volume of the supernatant in the tube is measured.
- The PMV is calculated as:

$$\text{PMV (\%)} = [(1\text{ml} - \text{Volume of supernatant in ml}) / (1\text{ml})] * 100$$

Result

The PMV was determined as discussed above in each of the samples collected from the fermenter.

4.3 MEASUREMENT OF VISCOSITY

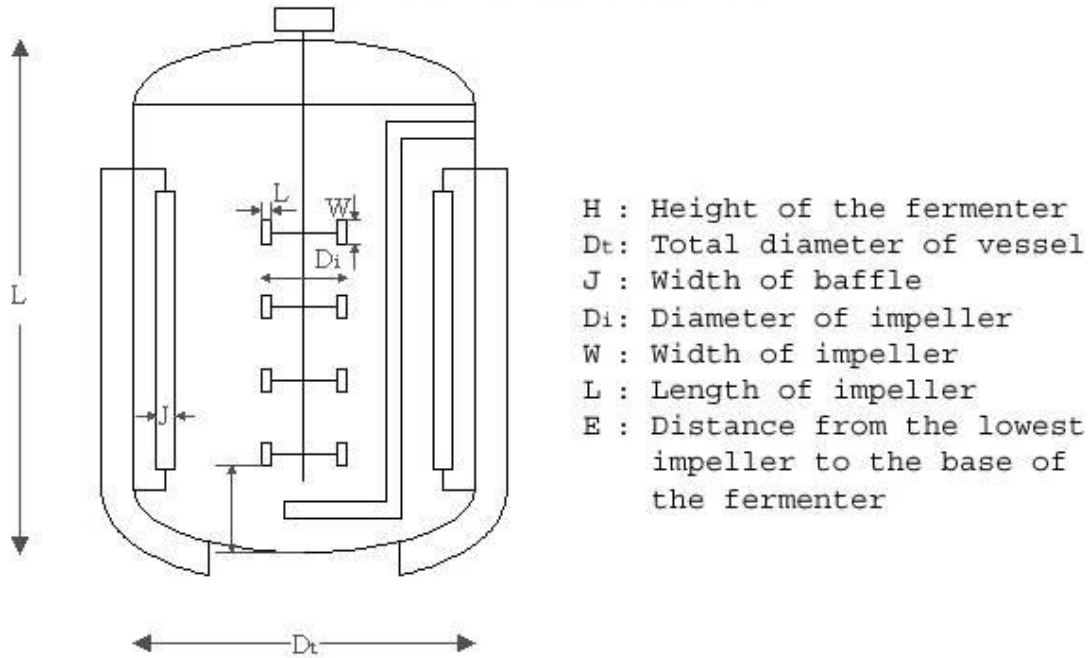
The viscosity of the broth sample was measured using a Brookfield Model DV – II + Viscometer using Spindle No.4.

4.4 MEASUREMENT OF pH

The pH of the broth sample was measured using Control Dynamics digital pH – meter with a glass electrode.

5.0 DESIGN OF FERMENTER

FERMENTER DESIGN



The most important fact to be considered in studying the design of a fermenter is that we may calculate the various physical dimensions of the fermenter and its components using standard design considerations. Most of these parameters are practically independent of the nature of the fermentation process. Once the fermenter is designed, it is vital to optimize the operating parameters (aeration and agitation rates) to suit the process being carried out.

Volume of the fermenter (V) = Volume of the cylinder + Volume of the 2 hemispherical heads

$$V = \pi R^2 H + 2 \times \frac{2}{3} \times \pi R^3$$

Assume $H = 3D_t$.

$$\begin{aligned} V &= \Pi D_t^2 \times \frac{H}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8} \\ &= \Pi D_t^2 \times \frac{3D_t}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8} \end{aligned}$$

Volume of the fermenter (V) = 200 liters = 0.2 m³

Therefore,

$$\Pi D_t^2 \times \frac{3D_t}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8} = 0.2 \text{ m}^3$$

$$\frac{11}{12} \times \Pi D_t^3 = 0.2 \text{ m}^3$$

$$D_t^3 = 0.0695 \text{ m}^3$$

$$D_t = 0.4110 \text{ m}$$

$$H = 3D_t = 3 \times 0.4110 = 1.2331 \text{ m}$$

Diameter of vessel (D_t) = 0.4110 m

Actual height $H_{\text{actual}} = 1.2 \times H = 1.4798 \text{ m}$

Thickness:

$$T = \frac{P \times D_t}{2 \times F \times J'} + C$$

$$P = 1.2 \text{ Kg/Cm}^2 = 0.11768 \text{ N/mm}^2$$

$$D_t = 411 \text{ mm}$$

$$F = 95 \text{ N/mm}^2$$

$$J' = 0.85$$

$$C = 1.5 \text{ mm}$$

$$T = \frac{0.11768 \times 411}{2 \times 95 \times 0.85} + 1.5$$

$$T = 1.7995 \text{ mm}$$

$$T \approx 2 \text{ mm}$$

Agitator Design

$$\begin{aligned} \text{No. of impellers} &= \frac{H \times \text{Broth Specific Gravity}}{D_t} \\ &= \frac{1.4798 \times 1.01}{0.411} = 3.636 \approx 4 \end{aligned}$$

$$\text{No. of blades} = 6$$

$$\text{Diameter of the impeller} = 0.4 \times D_t = 0.4 \times 0.411 = 0.164 \text{ m}$$

$$D_i = 0.164 \text{ m}$$

$$\text{Distance between the impellers} = D_i = 0.164 \text{ m}$$

$$\frac{E}{D_t} = \frac{1}{3}$$

$$E = D_t / 3 = 0.411 / 3 = 0.137 \text{ m} = 137 \text{ mm}$$

$$\frac{W}{D_i} = \frac{1}{5}$$

$$W = D_i / 5 = 0.164 / 5 = 0.0328 \text{ m} = 32.8 \text{ mm}$$

$$\frac{L}{D_i} = \frac{1}{4}$$

$$L = D_i / 4 = 0.164 / 4 = 0.041 \text{ m} = 41 \text{ mm}$$

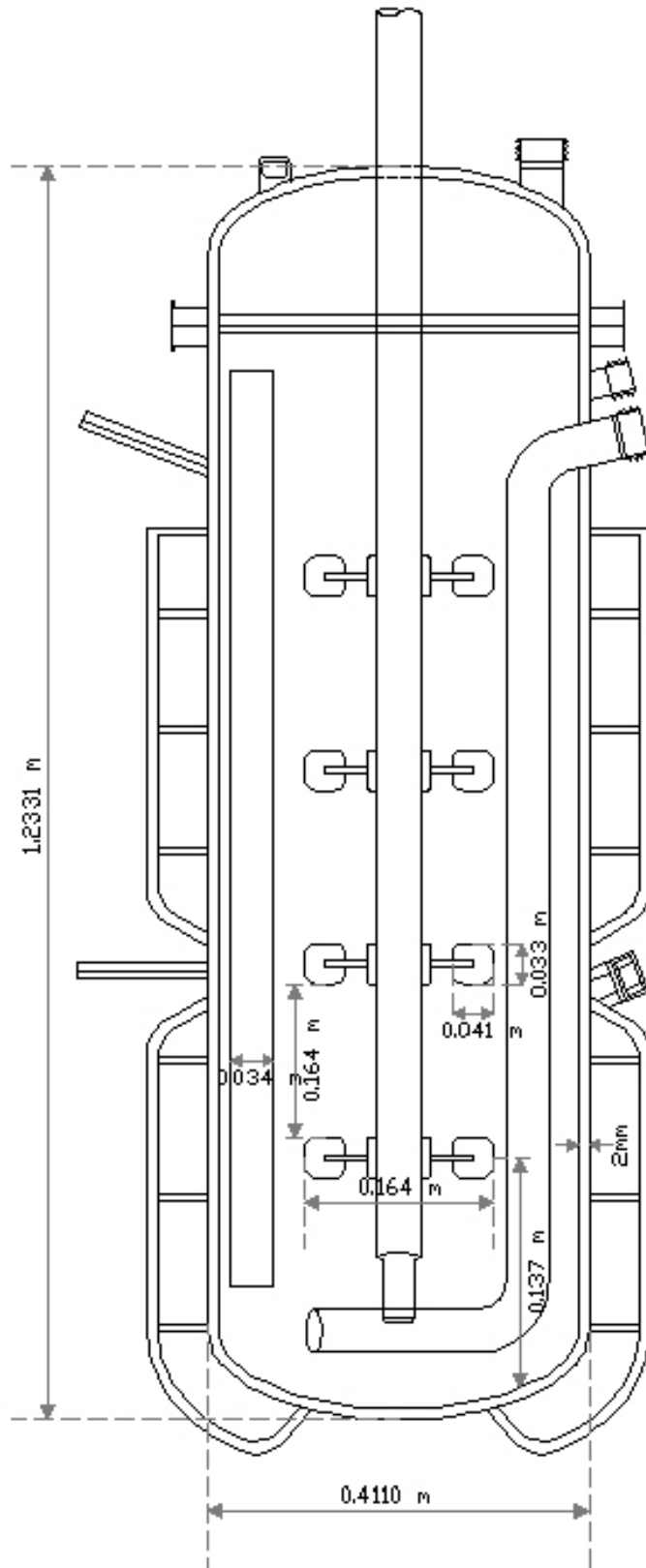
Width of the baffle

$$\frac{J}{D_t} = \frac{1}{12}$$

$$J = D_t / 12 = 34.3 \text{ mm}$$

No. of baffles = 4

FERMENTER – CUT AWAY DIAGRAM



6.0 SCALE-UP OF FERMENTERS

It is not possible to scale up fermenters based on geometrical considerations such as the height to diameter ratio as the conditions would change dramatically during scale-up with regard to surface aeration and wall growth. The physical conditions in a large fermenter can never duplicate those in a small fermenter if geometric similarity is maintained. Therefore we may use any of the following criteria for effective scale-up:

- a. Scale-up based on constant power input (P_o/V)
- b. Constant liquid circulation rate inside the vessel (pumping rate of impeller per unit volume, Q/V)
- c. Constant shear at impeller tip (ND_i)
- d. Constant Reynolds number ($ND_i^2\rho/\mu$)

We must note that $P \propto N^3D_i^5$, $V \propto D_i^3$, $Q \propto ND_i^3$, $P/V \propto N^3D_i^2$, and $Q/V \propto N$. Thus fixing N and D_i fixes all other quantities. Since these quantities have different dependencies on N and D_i , there will be a change in the physical environment of the cells within the reactor during scale-up. As a result different scale-up rules may give dramatically different results.

Constant Power Input Per Unit Volume

To use this scale-up rule we must make a distinction between gassed and non-gassed systems. The relation between gassed and non-gassed systems was expressed as follows by Michel and Miller:

$$\frac{P_g}{P_o} = \text{Constant} \times \frac{(N^{0.5}D^{1.5})}{Q^{0.25}}$$

where N is the agitation rate, D is the vessel diameter, Q is the volumetric gas flow rate, P_o is non-gassed power and P_g is the gassed power.

$$\frac{P_g}{D^3} = \frac{P_o}{D^3} \times \text{Constant} \times \frac{(N^{0.5} D^{1.5})}{Q^{0.25}}$$

i.e.,

$$\frac{P_g}{D^3} = N_p N^3 D^2 \times \text{Constant} \times \frac{(N^{0.5} D^{1.5})}{Q^{0.25}}$$

$$\frac{P_g}{D^3} = \text{Constant} \times \frac{(N^{3.5} D^{3.5})}{Q^{0.25}}$$

For scale-up we must maintain constant P_g/V , i.e. P_g/D^3 must be kept constant.

Thus we arrive at the following equation for scale-up:

$$\frac{(N^{3.5} D^{3.5})}{Q^{0.25}} = \text{constant}$$

Or

$$\frac{(N_1^{3.5} D_1^{3.5})}{Q_1^{0.25}} = \frac{(N_2^{3.5} D_2^{3.5})}{Q_2^{0.25}}$$

Thus we arrive at the following equation relating the impeller speeds of two bioreactors.

$$N_1 = N_2 \times \left(\frac{D_2}{D_1}\right) \times \left(\frac{Q_1}{Q_2}\right)^{0.071}$$

6.1 SCALE UP OF A 200 LITRE FERMENTER TO 1500 LITERS

$$\frac{H}{D_t} = \text{Constant}$$

Therefore,

$$(H/D_t)_{200} = (H/D_t)_{1500} \quad (\text{e 3.1})$$

Assuming $H = 3D_t$,

$$V = \Pi D_t^2 \times \frac{3D_t}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8}$$

$$\Pi D_t^2 \times \frac{3D_t}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8} = 1.5 \text{ m}^3$$

Solving the above equation, we get $D_t = 0.8538 \text{ m}$

Substituting this value in equation (e 3.1), we get:

$$1.2331/0.4110 = H_{1500}/0.8538$$

$$H_{1500} = 2.5616$$

Similarly,

$$\frac{E}{D_t} = \text{Constant}$$

$$(E/D_t)_{200} = (E/D_t)_{1500}$$

$$0.137/0.411 = E_{1500}/0.8538$$

$$E_{1500} = 0.2846 \text{ m}$$

$$D_i = 0.4 \times D_t$$

$$D_{i(1500)} = 0.4 \times 0.8538 = 0.3415 \text{ m}$$

$$\frac{W}{D_i} = \text{Constant}$$

$$(W/D_i)_{200} = (W/D_i)_{1500}$$

$$0.0328/0.164 = W_{1500}/0.3415$$

$$W_{1500} = 0.0683 \text{ m}$$

$$\frac{L}{D_i} = \text{Constant}$$

$$(L/D_i)_{200} = (L/D_i)_{1500}$$

$$0.041/0.164 = L_{1500}/0.3415$$

$$L_{1500} = 0.0854 \text{ m}$$

$$\frac{J}{D_t} = \text{Constant}$$

$$(J/D_t)_{200} = (J/D_t)_{1500}$$

$$0.0343/0.411 = J_{1500}/.8538$$

$$J = 0.0713 \text{ m}$$

6.2 SCALE UP OF A 200 LITRE FERMENTER TO 9000 LITRES

$$\frac{H}{D_t} = \text{Constant}$$

Therefore,

$$(H/D_t)_{200} = (H/D_t)_{9000} \quad (\text{e 4.1})$$

Assuming $H = 3D_t$,

$$V = \Pi D_t^2 \times \frac{3D_t}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8}$$

$$\Pi D_t^2 \times \frac{3D_t}{4} + \frac{4}{3} \times \Pi \frac{D_t^3}{8} = 9 \text{ m}^3$$

Solving the above equation, we get $D_t = 1.4620 \text{ m}$

Substituting this value in equation (e 4.1), we get:

$$1.2331/0.4110 = H_{9000}/1.4620$$

$$H_{9000} = 4.3864$$

Similarly,

$$\frac{E}{D_t} = \text{Constant}$$

$$(E/D_t)_{200} = (E/D_t)_{9000}$$

$$0.137/0.411 = E_{9000}/1.4620$$

$$E_{9000} = 0.4873 \text{ m}$$

$$D_i = 0.4 \times D_t$$

$$D_{i(9000)} = 0.4 \times 1.4620 = 0.5848 \text{ m}$$

$$\frac{W}{D_i} = \text{Constant}$$

$$(W/D_i)_{200} = (W/D_i)_{9000}$$

$$0.0328/0.164 = W_{9000}/0.5848$$

$$W_{9000} = 0.1170 \text{ m}$$

$$\frac{L}{D_i} = \text{Constant}$$

$$(L/D_i)_{200} = (L/D_i)_{9000}$$

$$0.041/0.164 = L_{9000}/0.5848$$

$$L_{9000} = 0.1462 \text{ m}$$

$$\frac{J}{D_t} = \text{Constant}$$

$$(J/D_t)_{200} = (J/D_t)_{9000}$$

$$0.0343/0.411 = J_{9000}/1.462$$

$$J = 0.1220 \text{ m}$$

7.0 RESULTS AND DISCUSSION

7.1 TABULATION GRAPHS AND RESULTS

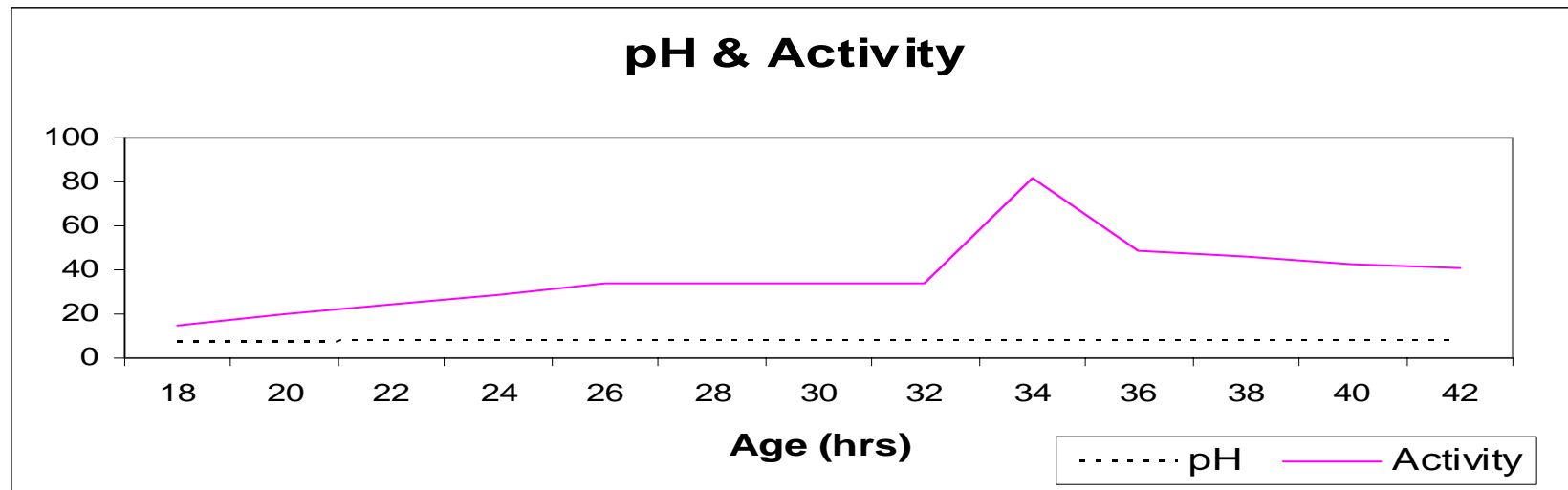
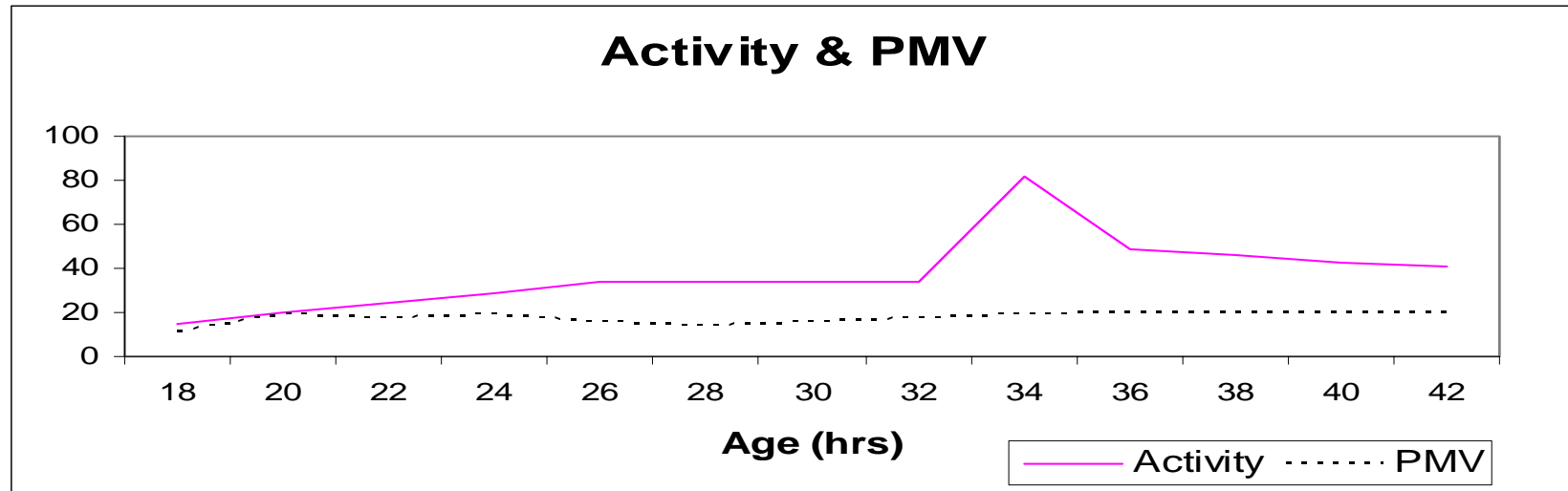
7.1.1 CONTROL BATCH

Speed **120 rpm**
Aeration **18 Nm³/hr**

Age hrs	Temperature °C	Back Pr. Kg/cm²	Viscosity cP	pH	Activity IU	PMV %
BI	28	0.50		7.17		
AI	28	0.50		7.10		
2	28	0.50	18.00	7.12		9.84
4	28	0.50	18.00	7.15		9.00
6	29	0.50	18.00	7.12		10.26
8	28	0.50	22.00	7.09		11.24
10	28	0.50	20.00	7.07		8.24
12	28	0.50	20.00	7.09		9.35
14	28	0.50	20.00	7.13		12.42
16	30	0.50	20.00	7.13		14.56
18	29	0.50	22.00	7.16	15.00	11.56
20	28	0.50	22.00	7.28	20.00	19.18
22	28	0.50	22.00	7.42	24.00	17.19
24	28	0.50	22.00	7.54	29.00	19.17
26	29	0.50	26.00	7.60	33.70	15.77
28	28	0.50	32.00	7.66	33.70	13.59
30	28	0.50		7.68	33.60	15.47
32	28	0.50		7.73	33.60	17.72
34	29	0.50	26.00	7.75	82.00	19.36
36	28	0.50	26.00	7.82	49.00	19.72
38	29	0.50	26.00	7.85	46.00	19.98
40	29	0.50		8.12	42.70	20.18
42	28	0.50		8.14	41.00	20.38

Microscopic Observation: Sterile

CONTROL BATCH



Results:

Appreciable activity was observed starting from 18 hours after inoculation. The activity reached a peak value of 82 IU at 34 hours. It declined to 45 – 50 IU and remained within that range for the next 6 hours, after which it dropped to 41 IU at 42 hours.

The pH declined from an initial value of 7.17 to 7.09 in the first 12 hours. It began to increase at about the same time as activity and the upward trend was observed till the end. The final value of pH reached was 8.14, which amounts to a net increase in pH by 0.97 over the entire batch.

The PMV increased initially from 9.84% to 19.17 in the first 24 hours. It then dropped to 13.59% over the next 4 hours and the increased gradually to 20%.

7.1.2 AGITATION STUDIES

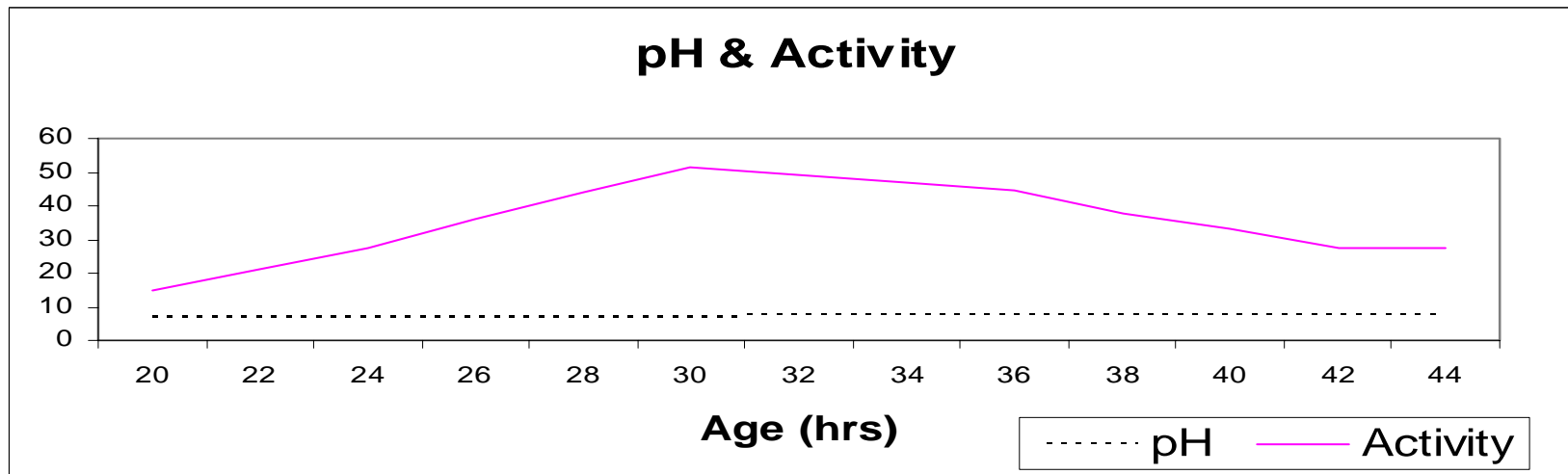
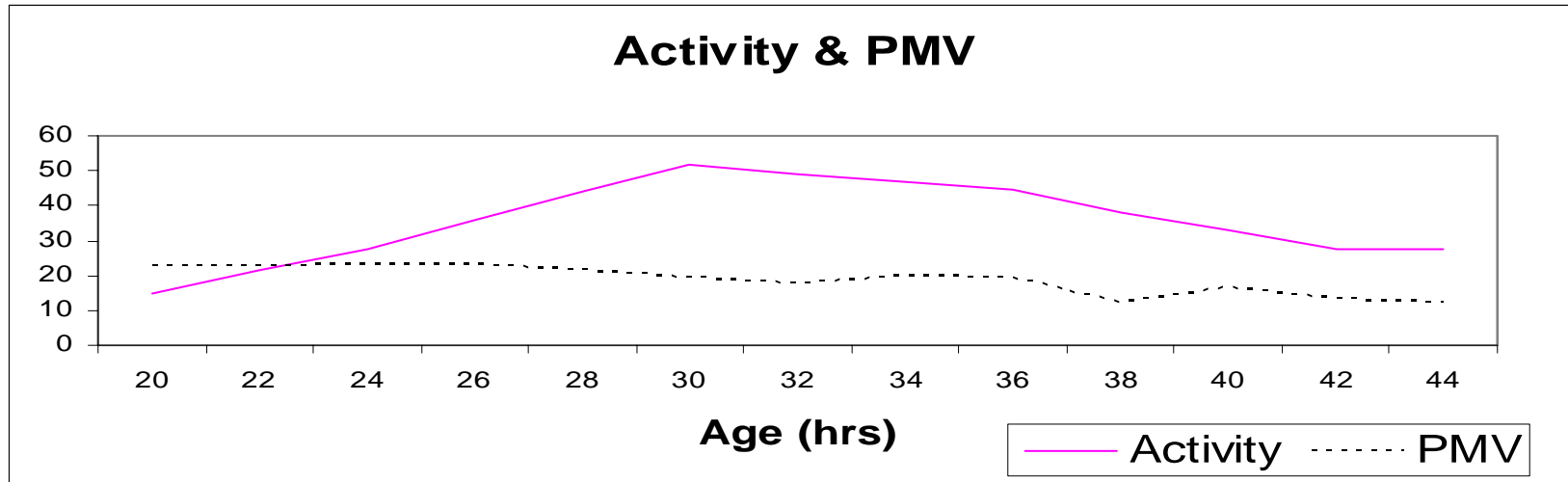
7.1.2.1 EXPERIMENTAL BATCH 1

Speed **60 rpm**
Aeration **18 Nm³/hr**

Age Hrs	Temperature °C	Back Pr. Kg/cm²	Viscosity cP	pH	Activity IU	PMV %
BI	28	0.50		6.80	-	
AI	28	0.50	18.00	6.73	-	8.57
2	28	0.50	18.00	6.75	-	7.76
4	28	0.50	18.00	6.73	-	6.90
6	29	0.50	16.00	6.73	-	10.79
8	28	0.50	22.00	6.65	-	8.06
10	28	0.50	26.00	6.57	-	19.90
12	28	0.50		6.54	-	20.46
14	28	0.50	30.00	6.53	-	23.67
16	29	0.50		6.62	-	
18	29	0.50	54.00	6.75	-	20.52
20	29	0.50	52.00	7.00	15.00	22.52
22	28	0.50	40.00	6.94	21.20	22.75
24	28	0.50	36.00	6.97	27.60	22.90
26	29	0.50	34.00	6.93	36.00	22.85
28	28	0.50	36.00	6.91	44.00	21.46
30	28	0.50	36.00	7.01	51.70	19.34
32	28	0.50	34.00	7.20	49.00	17.46
34	29	0.50	38.00	7.22	47.00	19.82
36	28	0.50		7.32	44.50	19.20
38	28	0.50		7.34	38.00	12.36
40	30	0.50	38.00	7.54	33.00	16.66
42	29	0.50		7.63	27.60	13.44
44	28	0.50	34.00	7.66	27.60	12.36

Microscopic Observation: Sterile

Agitation Studies – Experimental Batch 1



Results:

Activity was appreciable after 20 hours and reached a peak value of 51.7 IU at 30 hours. Activity was considerably high (40 – 50 IU) between 30 and 36 hours. Afterwards, it declined steadily to 27.6 IU by 44 hours.

The pH decreased from 6.8 to 6.62 over the first 16 hours. It then increased gradually, reaching a maximum value of 7.66 at 44 hours. A net increase in pH of 0.93 was observed over the entire batch

The PMV decreased by 1.67% during the first 6 hours. It increased sharply to 19.90% after 8 hours and remained close to 20% ($\pm 2\%$) up to 32 hours. It dropped slightly at 32 hours, increased again at 34 hours and finally decreased to 12.36% by 44 hours.

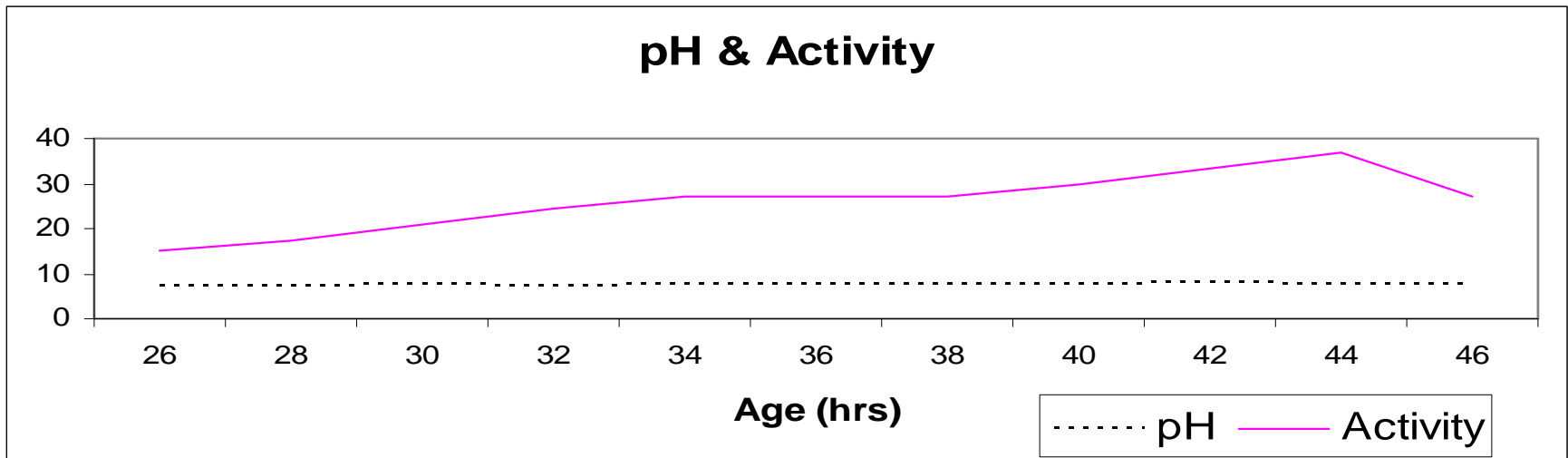
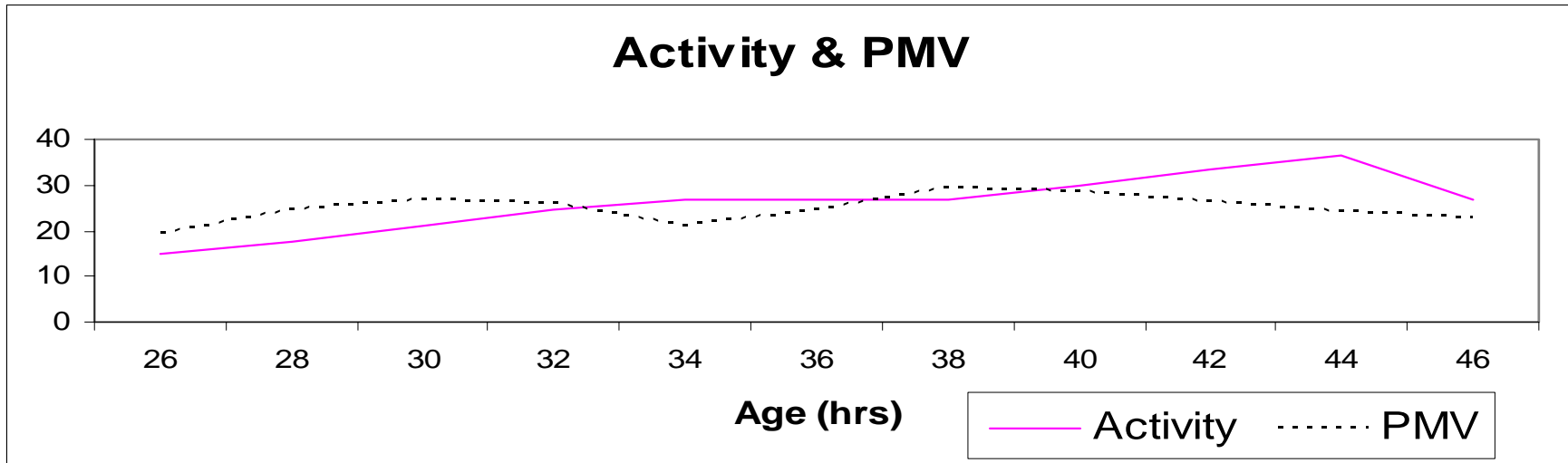
7.1.2.2 EXPERIMENTAL BATCH 2

Speed **180 rpm**
Aeration **18 Nm³/hr**

Age Hrs	Temperature °C	Back Pr. Kg/cm²	Viscosity cP	pH	Activity IU	PMV %
BI	28	0.50		6.80		
AI	28	0.50		6.72		
2	29	0.50	18.00	6.82		8.94
4	28	0.50	20.00	6.86		8.86
6	29	0.50	20.00	6.81		7.89
8	28	0.50	20.00	6.74		15.30
10	28	0.50	20.00	6.72		13.04
12	28	0.50	20.00	6.75		20.51
14	28	0.50	21.00	6.75		19.21
16	29	0.50	22.00	6.89		23.19
18	30	0.50	22.00	7.17		21.76
20	28	0.50	21.00	7.12		21.99
22	28	0.50	24.00	7.09		22.41
24	28	0.50	20.00	7.07		21.83
26	28	0.50	24.00	7.10	15.00	19.39
28	29	0.50	22.00	7.28	17.50	24.66
30	28	0.50	24.00	7.35	21.00	26.90
32	28	0.50	24.00	7.32	24.50	25.98
34	28	0.50	28.00	7.53	27.00	21.08
36	28	0.50	28.00	7.68	27.00	24.56
38	28	0.50	24.00	7.71	27.00	29.42
40	29	0.50	22.00	7.76	30.00	28.75
42	28	0.50	20.00	7.89	33.50	26.46
44	29	0.50		7.67	36.70	23.98
46	28	0.50	22.00	7.63	27.00	22.76

Microscopic Observation: Sterile

Agitation Studies – Experimental Batch 2



Results:

The activity was appreciable only after 26 hours and it reached a peak value of 36.7 IU at 44 hours.

The pH remained between 6.7 and 6.8 during the initial 14 hours. It increased starting from 16 hours and reaching a maximum value of 7.89 at 42 hours. It then declined slightly to 7.63 over the next 4 hours. The net increase in pH over the entire batch was 0.91.

The PMV decreased slightly in the first 6 hours. It increased quickly to 20.51% over the next 4 hours. After that, it increased with some fluctuations, reaching 24.66% at 28 hours. It remained between 25-26% for the next 8 hours. It increased again to reach a peak value of 29.42% at 38 hours and started decreasing gradually afterwards.

Comparing the two experimental batches run at 180 rpm and 60 rpm, it is seen that the enzyme activity is significantly higher at 60 rpm and that the rise in activity is also faster at 60 rpm.

7.1.3 AERATION STUDIES

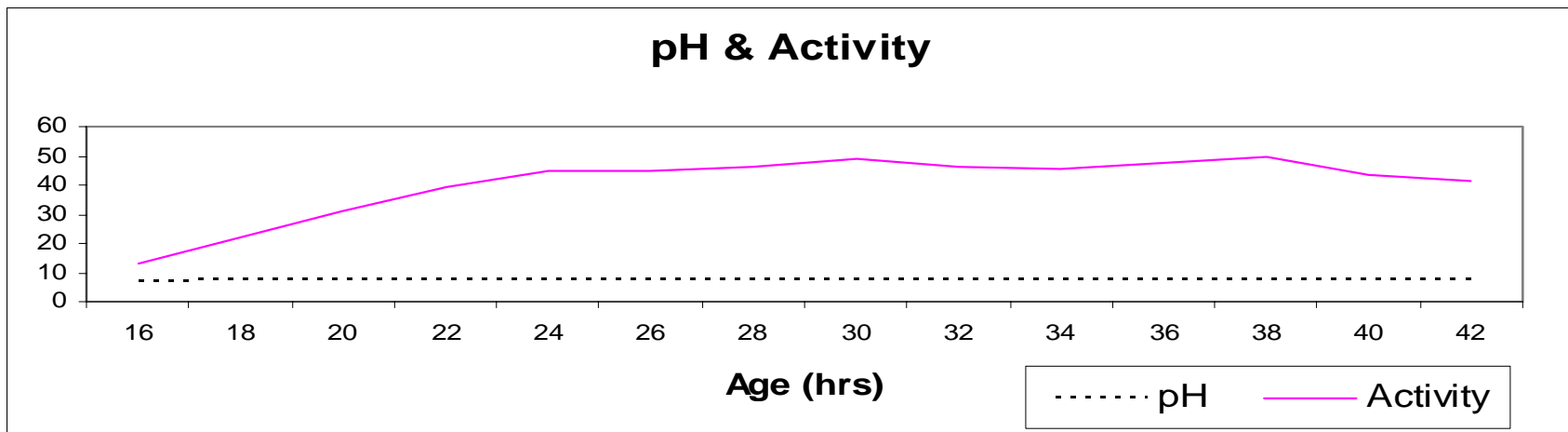
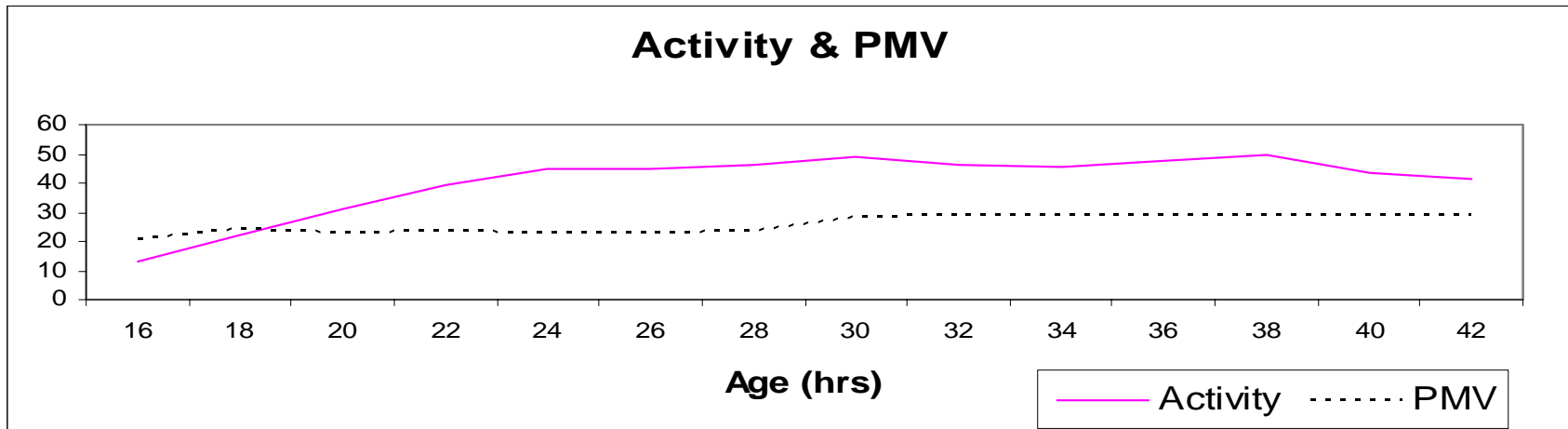
7.1.3.1 EXPERIMENTAL BATCH 1

Speed 120 rpm
Aeration 14.4 Nm³/hr

Age hrs	Temperature °C	Back Pr. Kg/cm ²	Viscosity cP	pH	Activity IU	PMV %
BI	28	0.50		6.98		
AI	28	0.50		6.92		
2	28	0.50	20	6.97		4.66
4	28	0.50	20	6.95		6.72
6	28	0.50	20	6.89		10.25
8	28	0.50	20	6.90		9.21
10	28	0.50	22	6.83		11.01
12	28	0.50	26	6.86		19.82
14	28	0.50	38	6.92		23.31
16	28	0.50	40	7.22	13.00	20.45
18	28	0.50	44	7.35	22.00	23.81
20	28	0.50	44	7.47	31.20	22.51
22	28	0.50	38	7.46	39.00	23.14
24	28	0.50	34	7.45	45.00	22.89
26	28	0.50	34	7.44	45.00	22.98
28	28	0.50	32	7.50	46.00	23.77
30	28	0.50	28	7.50	49.00	28.62
32	28	0.50	30	7.55	46.50	28.71
34	28	0.50	30	7.58	45.60	28.93
36	28	0.50		7.62	47.80	29.18
38	28	0.50	28	7.70	49.90	29.01
40	28	0.50	29	7.84	43.60	28.89
42	28	0.50	29	7.81	41.20	28.78

Microscopic Observation: Sterile

Aeration Studies - Experimental Batch 1



Results:

Activity was observed to be appreciable starting from 16 hours and reached a peak of 49 IU at 30 hours (the absolute maximum value was 49.9 IU at 38 hours). Between 24 and 38 hours, activity remained 45-50 IU, after which activity began to decline.

The pH decreased from 6.98 to 6.83 during the first 10 hours. It began to increase gradually afterwards reaching 7.47 at 20 hours. It remained nearly constant for the next 10 hours with very minor fluctuations. It began to increase again afterwards, reaching a maximum value of 7.84. There was a net increase of 0.89 in the pH during the batch.

The PMV increased gradually from 4.66% to 11.01% in the first 10 hours. It increased sharply to 19.82 at 12 hours and continued to increase steadily afterwards. It reached a maximum of around 29% and remained nearly constant thereafter.

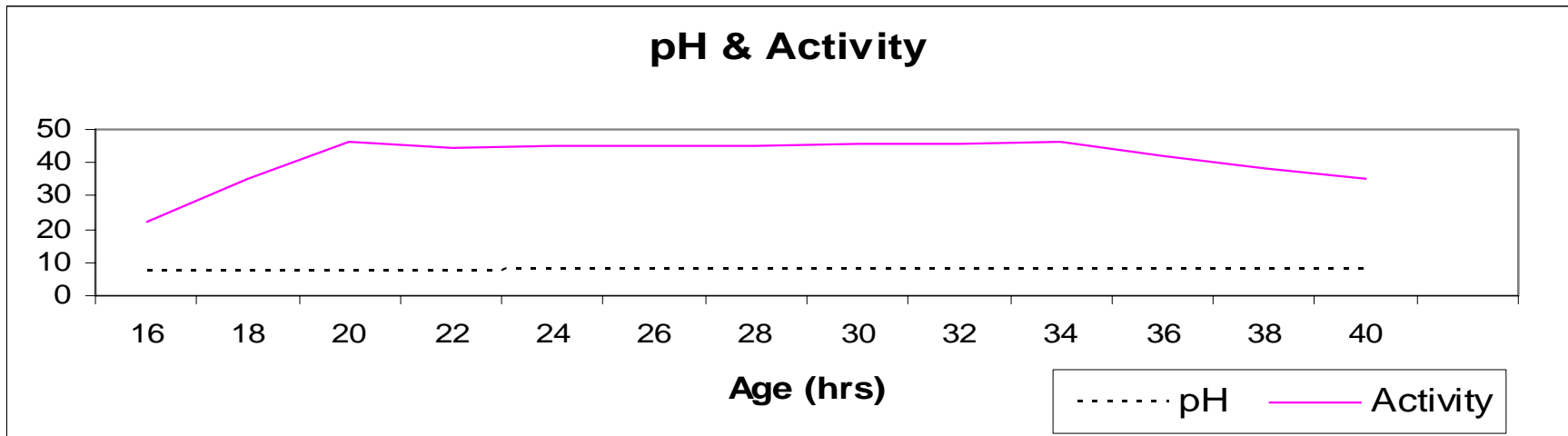
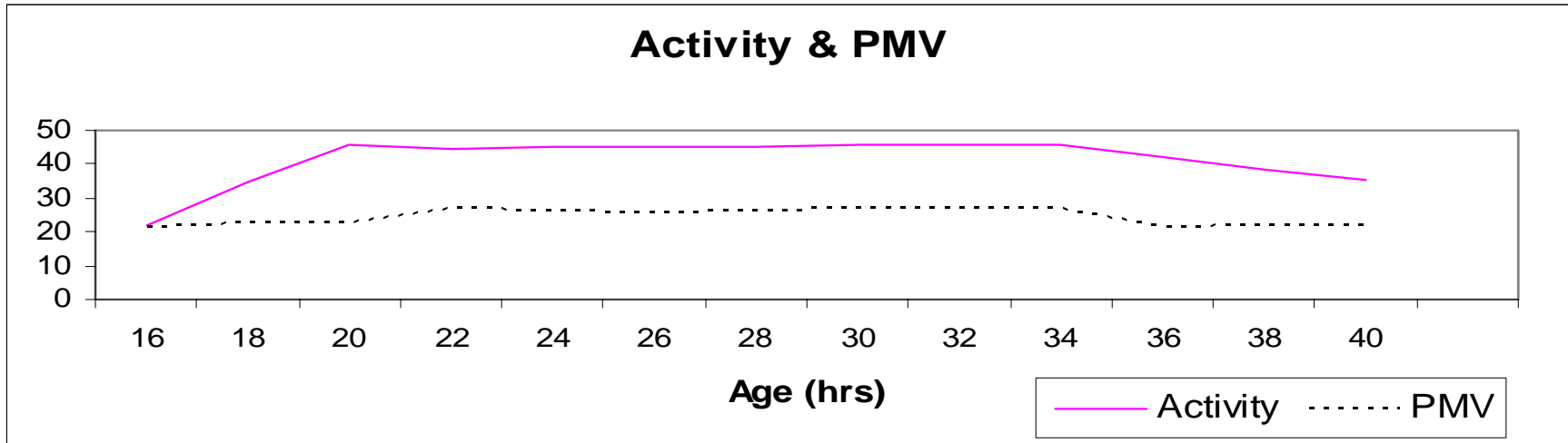
7.1.3.2 EXPERIMENTAL BATCH 2

Speed **120 rpm**
Aeration **10.8 Nm³/hr**

Age hrs	Temperature °C	Back Pr. Kg/cm²	Viscosity cP	pH	Activity IU	PMV %
BI	28	0.50		6.95		
AI	28	0.50		6.91		
2	29	0.50	18	6.93		10.20
4	28	0.50	18	6.89		14.36
6	28	0.50	18	6.90		13.98
8	29	0.50	20	6.73		13.71
10	30	0.50	20	6.78		14.21
12	29	0.50	48	7.11	6.15	28.34
14	28	0.50	48	7.18		27.61
16	28	0.50	52	7.38	22.05	21.50
18	28	0.50	60	7.53	35.00	22.57
20	28	0.50	72	7.55	46.00	22.41
22	28	0.50	76	7.64	44.70	26.74
24	29	0.50	58	7.86	45.05	26.04
26	28	0.50	62	7.97	45.14	25.82
28	28	0.50	58	7.90	45.30	25.98
30	28	0.50	60	7.97	45.44	26.82
32	28	0.50	64	7.95	45.82	26.75
34	28	0.50	64	7.91	46.03	26.75
36	29	0.50	52	7.98	42.16	21.34
38	28	0.50	50	8.07	38.34	22.18
40	28	0.50	42	8.12	35.26	22.04

Microscopic Observation: Sterile

Aeration Studies - Experimental Batch 2



Results:

Appreciable activity was observed after 12 hours. It reached a peak value of 46 IU at 20 hours and remained close to 45 IU up to 34 hours, after which it began to decline gradually.

The pH decreased from 6.95 to 6.73 in the first 8 hours. Afterwards it increased gradually, reaching 7.97 at 26 hours. It fluctuated close to 7.95 for the next 10 hours before increasing again to reach a maximum of 8.12 at 40 hours. The net pH increase observed during the batch was 1.21.

After increasing gradually by 4% in the initial 10 hours, the PMV suddenly rose to 28.34% at 12 hours. It then declined to 21.5% over the next 4 hours and remained close to 22.5 % for the subsequent 4 hour period. Afterwards it increased at 22 hours and fluctuated close to 26% up to 34 hours, before declining again.

Comparing the two experimental batches run at 14.4 Nm³/hr and 10.8 Nm³/hr, it is seen that the activity increased significantly faster at 10.8 Nm³/hr.

7.2 DISCUSSION OF RESULTS

Control Batch – 120 rpm, 18 Nm³/hr

Enzyme activity was observed over a pH range of 7.16 to 8.14. After 32 hours, the PMV increased steadily and remained close to 20%. Maximum activity was observed during the same period. Peak activity was observed at a pH of 7.75 and PMV of 19.36%.

The large spike in activity observed at 34 hours may be due to analytical error. However it may also be attributed to other physiological reasons. A sudden increase in back pressure from 0.5 Kg/cm² to around 2 Kg/cm² for about 5-10 minutes that occurred just a short while before the spike during a change over of compressors, could be of significance as the increase in pressure may have triggered release of enzymes.

Agitation Studies:

Experimental Batch 1 – 60 rpm, 18 Nm³/hr

Activity remained close to the maximum of 51.70 IU between 28 hours and 36 hours. The rise in activity was slightly quicker in comparison to the control batch. Also high activity was maintained for a longer period of time than in the control batch.

Activity was observed over a pH range of 7.0 to 7.66. The net increase in pH over the entire batch was similar to the control batch. Peak activity occurred at a pH of 7.01 (which is slightly lower than the control batch).

The period of high activity coincided with a period of high PMV. As activity began to decline, a similar trend was observed in the PMV. The maximum value of PMV was higher in comparison to the control batch. It was also reached faster and sustained for a longer period than in the control batch. Activity peaked at a PMV of 19.34% (which is almost the same as the control batch).

Experimental Batch 2 – 180 rpm, 18 Nm³/hr

The activity began to increase only after 26 hours, which was slower by 8 hours in comparison to the control batch. Also, the peak value of 36.70 IU was lower than the control batch value.

The PMV was considerably higher than the control batch and also increased at a faster rate. Yet the activity was lower than the control batch, which indicates that the conditions did not favor enzyme production. Also it was observed that the fungal pellets appeared to be sheared into loose mycelia indicating that the shear force due to agitation was too strong. This might have had a significant effect on enzyme production.

Activity was observed over a pH range of 7.10 to 7.63 and peaked at a pH of 7.67 (similar to the control batch). The PMV was observed to be 26.46 % when activity was at its maximum (7.1% higher than the control batch).

Aeration Studies:

Experimental Batch 1 – 120 rpm, 14.4 Nm³/hr

The activity began to increase after 16 hours, only 2 hours earlier than the control batch. However it increased at a faster rate and reached 45 IU by 24 hours. The activity remained stable and high for the next 14 hours which is significantly longer than the control batch.

The increase in PMV was faster than the control batch. The maximum PMV reached was higher by nearly 9% than in the control batch and also the PMV remained high for a much longer period of time. These indicate that the conditions were suitable for the fungus to grow. Also the period of high PMV coincided with the period of maximum activity.

Activity was observed over a pH range of 7.22 to 7.81 and maximum activity occurred at a pH of 7.70 (nearly same as in the control batch). When activity peaked, the PMV was at 29.01%, which is higher than the control batch value by over 9%.

Experimental Batch 2 – 120 rpm, 10.8 Nm³/hr

Activity began to increase after only 12 hours which is faster than the control batch by 6 hours. Also the rate of increase was much faster, with the activity crossing 45 IU by 20 hours. Activity remained stable at above 40 IU for the next 16 hours, much longer than the control batch.

The rate of increase and the maximum value of PMV (28.34%) were both higher in comparison to the control batch. The period of high PMV corresponded to the period of high activity.

Activity was observed over a pH range of 7.11 to 8.12 and peaked at a pH of 7.91 (only slightly higher than the control batch value). PMV was at 26.75% when peak activity was observed (higher than the control batch value by roughly 6%).

8.0 CONCLUSION

- It is inferred that an agitation rate of 60 rpm and an aeration rate of 10.8 Nm³/hr have both been independently found to be the most favorable parameters to achieve maximum enzyme production.
 - Advantages observed at 60 rpm:
 - Reduced energy expenditure for agitation
 - Possible saving of 6 hours running time over control batch
 - Slight improvement in activity
 - Advantages observed at 10.8 Nm³/hr:
 - Reduced energy expenditure for aeration
 - Possible saving of 12-14 hours running time over control batch
 - High activity maintained for a much longer period of time, which implies that scope exists for much higher activity to be reached by improving the process further.
- A 200 liter fermenter was designed and various design parameters were calculated. The results are given in Appendix 1.
- The 200 liter fermenter was scaled up to 1500 liters and 9000 liters. The results are given in Appendices 2 and 3 respectively.

9.0 SCOPE FOR FURTHER STUDIES

It is suggested that further studies be carried out with a combination of the settings suggested above, i.e. 60 rpm, 10.8 Nm³/hr.

Also, the long period of stagnant activity observed in the experimental batch run at 120 rpm and 10.8 Nm³/hr indicates that there is stagnation in nutrient utilization during that period. Increasing agitation or aeration at that time may be useful to achieve even higher activity.

10.0 APPENDIX 1

DESIGN SPECIFICATIONS OF FERMENTERS

Specification	200 liters	1500 liters	9000 liters
D_t	0.4110	0.8538	1.4620
H	1.2331	2.5616	4.3864
H_{act}	1.4798	3.0739	5.2637
D_i	0.1640	0.3415	0.5848
J	0.0343	0.0713	0.1220
W	0.0328	0.0683	0.1170
L	0.0410	0.0854	0.1462
E	0.1370	0.2846	0.4873

Note: All dimensions are in meters.

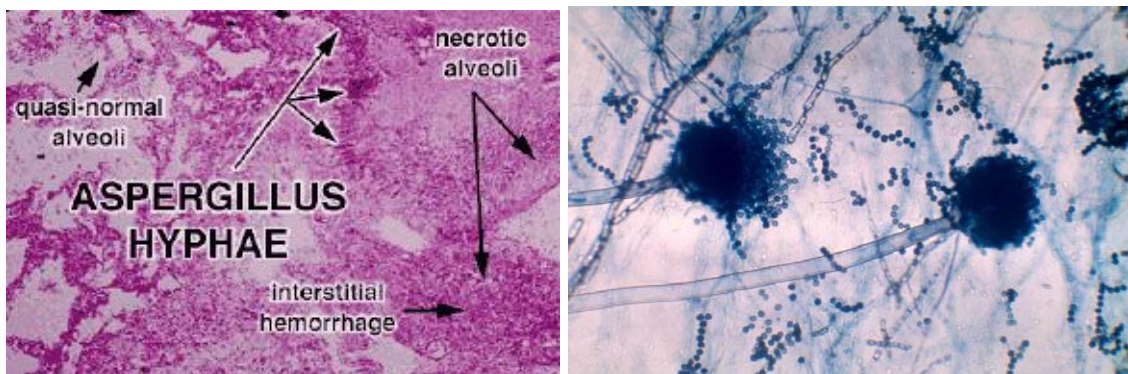
11.0 APPENDIX 2

PHOTOGRAPHS

Xylanase Molecule



Aspergillus – Microscopic Views



Laminar Flow Workbench



Shake Flasks on Orbital Shaker



Spectrophotometer

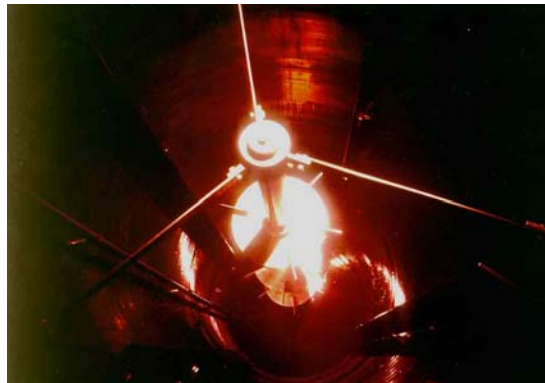


Laboratory Scale / Seed Fermenter



Production Fermenter

Agitator Inside View



12.0 BIBLIOGRAPHY

• BOOKS

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