DESIGN AND PREPARATION OF MEDIA FOR FERMENTATION

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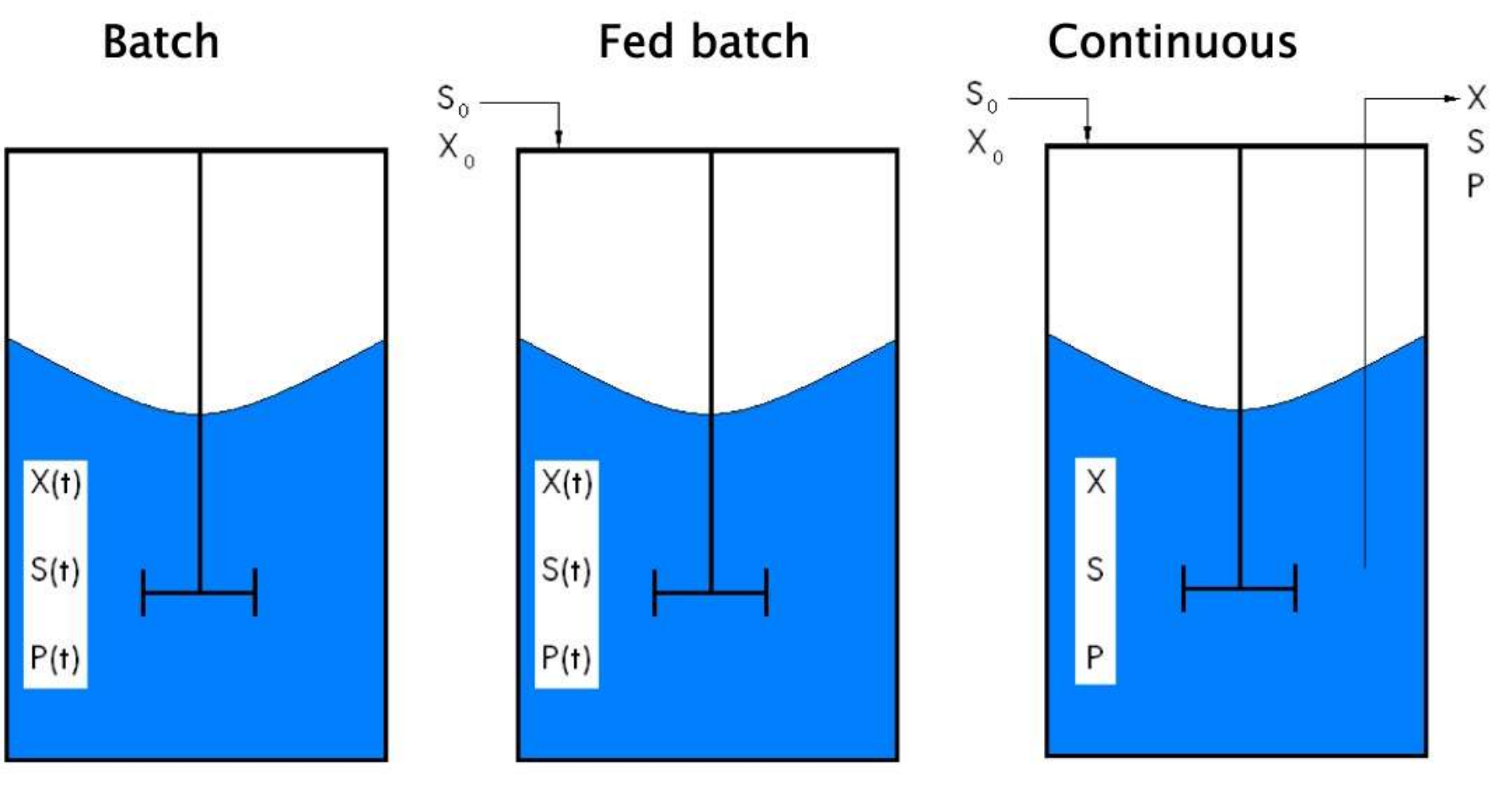
Fermentation

- **Fermentation is the process in which a substance breaks down into a simpler substance using organism.**
- **Its biochemical meaning relates to the generation of energy by the catabolism of organic compounds.**
- **Fermentation is a word that has many meanings for the microbiologist:**
- **1. Any process involving the mass culture of microorganisms, either aerobic or anaerobic.**
- **2. Any biological process that occurs in the absence of O2.**

TYPES OF FERMENTATION

- Batch fermentation
- Continuous fermentation
- Fed-batch fermentation



Submerged Liquid Fermentations

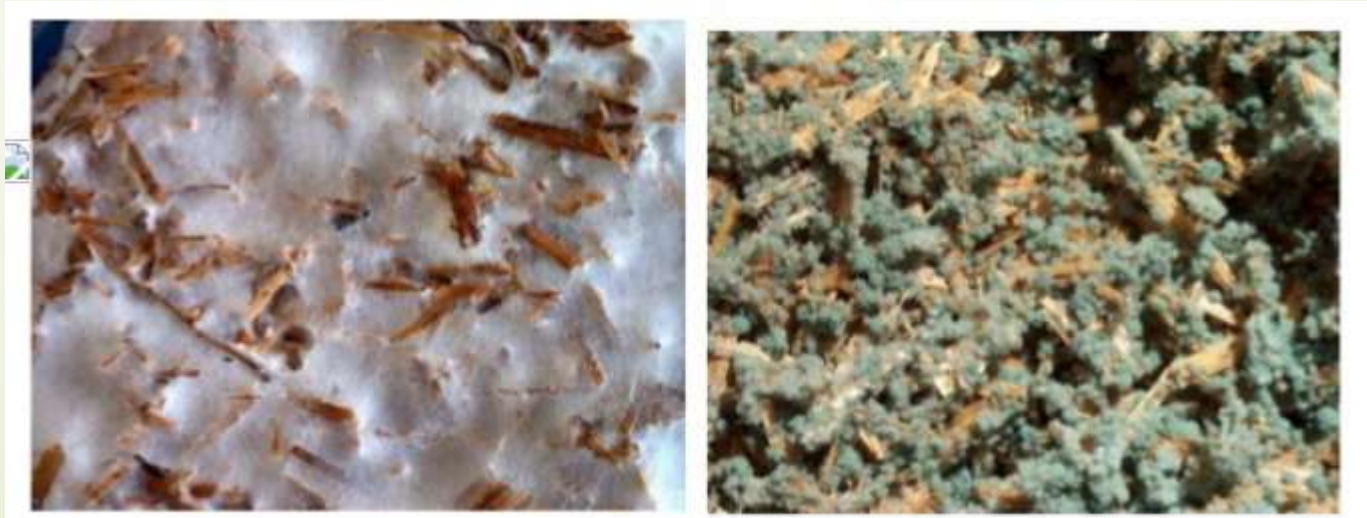
- Submerged liquid fermentations are traditionally used for the production of microbially derived enzymes. Submerged fermentation involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth.
- Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic metres. The fermentation media sterilises nutrients based on renewable raw materials like maize, sugars and soya.
- Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the carbon and nitrogen sources. Batch-fed and continuous fermentation processes are common. In the batch-fed process, sterilised nutrients are added to the fermenter during the growth of the biomass.

Cont..

- In the continuous process, sterilised liquid nutrients are fed into the fermenter at the same flow rate as the fermentation broth leaving the system. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation are measured and controlled to optimize the fermentation process.
- Next in harvesting enzymes from the fermentation medium one must remove insoluble products, e.g. microbial cells. This is normally done by centrifugation. As most industrial enzymes are extracellular (secreted by cells into the external environment), they remain in the fermented broth after the biomass has been removed.
- The enzymes in the remaining broth are then concentrated by evaporation, membrane filtration or crystallization depending on their intended application. If pure enzyme preparations are required, they are usually isolated by gel or ion exchange chromatography.

Solid-State Fermentation (SSF)

- SSF is used for the production of bioproducts from microorganisms under conditions of low moisture content for growth. The medium used for SSF is usually a solid substrate (e.g., rice bran, wheat bran, or grain), which requires no processing
- fewer problems due to contamination are observed.
- The power requirements are lower than submerged fermentation.
- Inadequate mixing, limitations of nutrient diffusion, metabolic heat accumulation, and ineffective process control renders SSF generally applicable for low value products with less monitoring and control



Growth of *Trichoderma sps.* on wheat straw in Solid State Fermentation

Solid-substrate culture	Submerged liquid culture
Culture medium is not free flowing	Culture medium is always free-flowing
Depth of medium is usually shallow except for a few bioreactors	Medium depth varies
Single water insoluble substrate provides carbon, nitrogen, minerals and energy	Different water-soluble sources of nutrients are used
Gradients in nutrient concentration are common	Nutrients are uniformly distributed throughout the fermentation
Water availability is just sufficient to sustain optimum growth of the culture	Water availability is abundant
Culture systems involves three phases, solid, liquid, and gaseous	Two phases, liquid and gaseous
Culture system is not aseptic beyond medium cooking	Whole system is always under aseptic conditions
Rigorous control of parameters is not required except for heat removal, oxygen supply and moisture	Rigorous control of all parameters during fermentation is essential
Inoculum ratio is always larger	Inoculum ratio is usually low
System may or may not involve agitation	Agitation is often essential
Fungal growth involves penetration of the hyphae deep into solid substrate particles	Fungal mycelial cells grow in the form of individual mycelium or mycelial pellets
Bacterial and yeast cells grow by adhering to solid particles	Bacterial and yeast cells are uniformly distributed throughout the liquid

- Micro-organisms used for fermentation process grow on or in growth medium which satisfies the nutritional needs of microbes.
- Complete analysis is needed to be done to establish the most favourable medium for the growth of the microbe used for fermentation.
- Formulating medium at lab scale can be done by adding main ingredients like water, carbon source, nitrogen source, minerals and other supplements in pure form and in required quantities is very easy which supports the growth of the microbe whereas, the same may not support the satisfactory growth of the same organism at industrial level.
- Following criteria need to be satisfied for the material to be treated as medium at industrial level.
- It should give maximum yield of product.
- It should give minimum yield of undesired product.
- It should be consistently available throughout the year.
- It should be cheap.

- But, at industrial level cane molasses, corn steep liquor, sugar beet juice which are inexpensive sources are utilised.
- On contrary, some sensitive fermentation makes use of glucose, sucrose and other carbohydrates in their pure form which ensures the purity and quality of the final product.
- Sometimes starch will be added to the medium for the specific production of amylases.
- At lab level, peptone or tryptone or beef extract which is a partially digested hydrolysate, which is utilised in synthesis of proteins, components of nucleic acids and other essential cellular components.
- But at industrial level it is supplemented with soy meal or ammonia or nitrate salts to supplement the nitrogen source. Other elements include growth factors, vitamins, anti-foaming agents, precursors, inducers chelating agents, trace elements such as Fe, Cu, Mn, Mo and Co, are added to the fermentation medium.
- Where growth factors, vitamins, precursors, inducers and trace elements directly supports the growth of microbe and anti-foaming agents are added to prevent the foam formation, in case of presence of higher concentrations of metal ions which is not preferable chelating agents are added.

Solid state fermentation for the production of industrial enzymes

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Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes. Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented products may be used directly as enzyme sources. This review focuses on the production of various industrial enzymes by SSF processes. Following a brief discussion of the microorganisms and the substrates used in SSF systems, and

employed for enzyme production in SSF systems. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. For example, it has been reported that while a strain of *Aspergillus niger* produced 19 types of enzymes, α-amylase was being produced by as many as 28 microbial cultures[3]. Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Generally, hydrolytic enzymes, e.g. cellulases, xylanases, pectinases, etc. are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth.

Table 2. Spectrum of microbial cultures employed for producton of various enzymes in solid state fermentation systems

Substrate	Microorganisms	Enzyme	References
Bagasse	*Trichoderma reesei + Aspergillus phoenicis*	Cellulases	22
Coconut coir pith	*A. niger*	Cellulases, β-glucosidase	23
Grapevine trimming dust	*Cerrena unicolor*	Cellulase, xylanase, ligninases	24
Rice husk	*Penicillium citrinum*	Cellulases	25
Rice husk	*Mesophilic fungi* (10 species)	Cellulase (FP)	26
Tea production waste	*Cerrena unicolor, Coriolus hirsutus, Pleurotus ostreatus*	CMCase, xylanase, laccase	27
Wheat bran	*A. niger*	Cellulase, xylanase, polygalacturonase	28
Cellulose, starch	*T. viride, A. niger*	Cellulase, amylase	29
Ligno-cellulosic materials	*Lentinula edodus*	Various enzymes	30
Bagasse	Strains of Basidiomycetes	Cellulase, ligninase	31
Cellulosics	*T. reesei*	Cellulase	32
Sweet sorghum silage, wheat straw	*Gliocladium* sp., *Trichoderma* sp., *Penicillium* sp.	Cellulase, xylanase	33
Agro-wastes	*A. niger*	Cellulase, β-glucosidase	34
Agro-wastes	*T. reesei*	Cellulase	35
Sugar beet pulp	*P. capsulatum*	Polysaccharide degrading enzymes	36
Wheat bran + rice straw, spent wheat bran	*Trichoderma* sp., *Botritis* sp., *A. ustus*, *Sporotrichum pulverulentum*	Cellulase, β-glucosidase, xylanase	37
Bagasse	*Polyporus* sp.	Cellulase, ligninase	38
Wheat straw	*Neurospora crassa*	CMCase, β-glucosidase	39
Rice straw, spent wheat bran	*Botritis* sp., *A. ustus, S. pulverulentum*	β-glucosidase, xylanase	40
Wheat bran	*T. reesei, S. pulverulentum*	Cellulase	41
Wheat straw + wheat bran	*T. harzianum*	Cellulase	42
Cellulosic wastes	*T. reesei*	Cellulase	43
Agro-wastes	*Spiecellum roseum*	CMCase	44
Bagasse, wheat bran, rice bran	*Aspergillus* sp.	Cellulase, β-glucosidase	45
8 ligno-cellulosic substrates	*Streptomyces* sp.	Cellulase	46
Agro-wastes	*Pestalotiopsis versicolor*	Cellulase	47
Wheat bran	*T. reesei*	Cellulase	48
Cellulosic wastes	*T. viride*	Cellulase	49
Agro-wastes	*Trichoderma* sp.	Cellulase	50

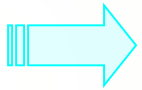
NUTRIENTS

- Most fermentations require liquid media, often referred to as broth; although some solid substrate fermentations (SSF) are operated.
- Fermentation media must satisfy all the nutritional requirements of the microorganism and fulfil the technical objectives of the process.
- All microorganisms require water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins plus oxygen if aerobic.
- The nutrients should be formulated to promote the synthesis of the target product, either cell biomass or a specific metabolite.
- In most industrial fermentation processes there are several stages where media are required. They may include several inoculum (starter culture) propagation steps, pilot scale fermentations and the main production fermentation. The technical objectives of inoculum propagation and the main fermentation are often very different, which may be reflected in differences in their media formulations.

Medium formulation

- Medium formulation is essential stage in manufacturing process

Carbon & Nitrogen other

Energy + sources + O_2 + nutrients  Biomass + products + CO_2 +H_2O +heat

- Elemental composition of microorganisms may be taken as guide

Elemental composition

Element	Bacteria	Yeast	Fungi
Carbon	50-53	45-50	40-63
Hydrogen	7	7	7
Nitrogen	12-15	7.5-11	7-10
Phosphorus	2-3	0.8-2.6	0.4-4.5
Sulphur	0.2-1.0	0.01-0.24	0.1-0.5
Potassium	1.0-4.5	1-4	0.2-2.5
Sodium	0.5-1.0	0.01-0.1	0.02-0.5
Calcium	0.01-1.1	0.1-0.3	0.1-1.4
Magnesium	0.1-0.5	0.1-0.5	0.1-0.5
Chloride	0.5	--	--
Iron	0.02-0.2	0.01-0.5	0.1-0.2

CARBON SOURCE

- A carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance. In most fermentations it also serves as the energy source.
- Molasses
- malted barley
- Starch and Dextrins
- Sulphite Waste Liquor
- Alkanes and Alcohols n-Alkanes
- Oils and fats

Factors influencing the carbon source

- Cost of the product
- rate at which it is metabolized
- geographical locations
- government regulations
- cellular yield coefficient

Nitrogen Sources

- Most industrial microbes can utilize both inorganic and organic nitrogen sources.
- Inorganic nitrogen may be supplied as ammonium salts, often ammonium sulphate and diammonium hydrogen phosphate, or ammonia. Ammonia can also be used to adjust pH of the fermentation.
- Organic nitrogen sources include amino acids, proteins and urea.
- Corn Steep Liquor
- Yeast Extracts
- Peptones
- Soya Bean Meal

Minerals

- All microorganisms require certain mineral elements for growth and metabolism. In many media, magnesium, phosphorous, potassium, sulphur, calcium and chlorine are essential components and must be added.
- Others such as cobalt, copper, iron, manganese, molybdenum and zinc are present in sufficient quantities in the water supplies and as impurities in other media ingredients.

Component	Range
*KH_2PO_4	1.0–4.0 (part may be as buffer)
$MgSO_4 \cdot 7H_2O$	0.25–3.0
KCl	0.5–12.0
$CaCO_3$	5.0–17.0
$FeSO_4 \cdot 4H_2O$	0.01–0.1
$ZnSO_4 \cdot 8H_2O$	0.1–1.0
$MnSO_4 \cdot H_2O$	0.01–0.1
$CuSO_4 \cdot 5H_2O$	0.003–0.01
$Na_2MoO_4 \cdot 2H_2O$	0.01–0.1

Chelators

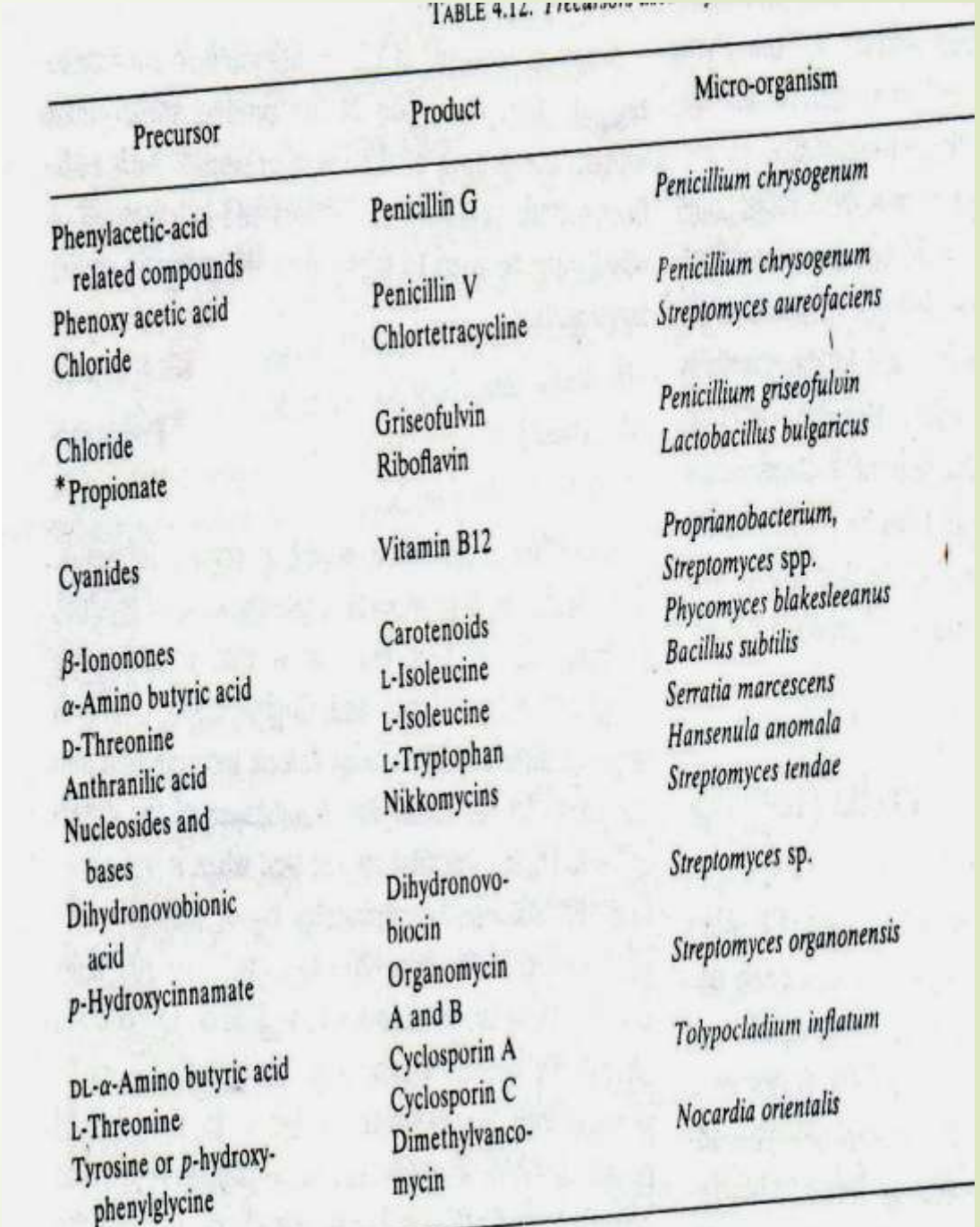
- Many media cannot be prepared without precipitation during autoclaving. Hence some chelating agents are added to form complexes with metal ions which are gradually utilised by microorganism
- Examples of chelators: EDTA, citric acid, polyphosphates etc.,
- It is important to check the concentration of chelators otherwise it may inhibit the growth.
- In many media these are added separately after autoclaving Or yeast extract, peptone complex with these metal ions

Vitamins and Growth Factors

- Many bacteria can synthesize all necessary vitamins from basic elements. For other bacteria, filamentous fungi and yeasts, they must be added as supplements to the fermentation medium.
- Most natural carbon and nitrogen sources also contain at least some of the required vitamins as minor contaminants

Precursors

- Precursors are defined as “substances added prior to or simultaneously with the fermentation which are incorporated without any major change into the molecule of the fermentation product and which generally serve to increase the yield or improve the quality of the product”.
- They are required in certain industrial fermentations and are provided through crude nutritive constituents, e.g., corn steep liquor or by direct addition of more pure compounds.



Precursor	Product	Micro-organism
Phenylacetic-acid	Penicillin G	*Penicillium chrysogenum*
related compounds		
Phenoxy acetic acid	Penicillin V	*Penicillium chrysogenum*
Chloride	Chlortetracycline	*Streptomyces aureofaciens*
Chloride	Griseofulvin	*Penicillium griseofulvin*
*Propionate	Riboflavin	*Lactobacillus bulgaricus*
Cyanides	Vitamin B12	*Proprianobacterium,* *Streptomyces spp.*
β-Iononones	Carotenoids	*Phycomyces blakesleeanus*
α-Amino butyric acid	L-Isoleucine	*Bacillus subtilis*
D-Threonine	L-Isoleucine	*Serratia marcescens*
Anthranilic acid	L-Tryptophan	*Hansenula anomala*
Nucleosides and	Nikkomycins	*Streptomyces tendae*
bases		
Dihydronovobionic	Dihydronovo-	*Streptomyces sp.*
acid	biocin	
p-Hydroxycinnamate	Organomycin	*Streptomyces organonensis*
	A and B	
DL-α-Amino butyric acid	Cyclosporin A	*Tolypocladium inflatum*
L-Threonine	Cyclosporin C	
Tyrosine or *p*-hydroxy-	Dimethylvanco-	*Nocardia orientalis*
phenylglycine	mycin	

Inducers and Elicitors

- If product formation is dependent upon the presence of a specific inducer compound or a structural analogue, it must be incorporated into the culture medium or added at a specific point during the fermentation.
- The majority of enzymes of industrial interest are inducible. Inducers are often substrates such as starches or dextrins for amylase.
- In plant cell culture the production of secondary metabolites, such as flavanoids and terpenoids can be triggered by adding elicitors.

Enzyme	Inducer	Micro-organism
α-Amylase	Starch	*Aspergillus* spp.
	Maltose	*Bacillus subtilis*
Pullulanase	Maltose	*Aerobacter aerogenes*
α-Mannosidase	Yeast mannans	*Streptomyces griseus*
Penicillin acylase	Phenylacetic acid	*Escherichia coli*
Proteases	Various proteins	*Bacillus* spp.
		Streptococcus spp.
		Streptomyces spp.
		Asperigillus spp.
		Mucor spp.
Cellulase	Cellulose	*Trichoderma viride*
Pectinases	Pectin (beet pulp, apple pomace, citrus peel)	*Aspergillus* spp.
Nitralase	Isovaleronitrile	*Rhodococcus rhodochrous*

Inhibitors

- Inhibitors are used to redirect metabolism towards the target product and reduce formation of other metabolic intermediates
- others halt a pathway at a certain point to prevent further metabolism of the target product.
- An example of an inhibitor specifically employed to redirect metabolism is sodium bisulphite

Product	Inhibitor	Main effect	Micro-organism
Glycerol	Sodium bisulphite	Acetaldehyde production repressed	*Saccharomyces cerivisiae*
Tetracycline	Bromide	Chlortetracycline formation repressed	*Streptomyces aureofaciens*
Glutamic acid	Penicillin	Cell wall permeability	*Micrococcus glutamicus*
Citric acid	Alkali metal/phosphate, pH below 2.0	Oxalic acid repressed	*Aspergillus niger*
Valine	Various inhibitors	Various effects with different inhibitors	*Brevibacterium roseum*
Rifamycin B	Di-ethyl barbiturate	Other rifamycins inhibited	*Nocardia mediterranei*
7-Chloro-6 demethyltetracycline	Ethionine	Affects one-carbon transfer reactions	*Streptomyces aureofaciens*

WATER

- All fermentation processes, except SSF, require vast quantities of water.
- Not only is water a major component of all media, but it is important for ancillary services like heating, cooling, cleaning and rinsing.
- A reliable source of large quantities of clean water, of consistent composition, is therefore essential.
- Assessing suitability of water
  - pH
  - dissolved salts
  - effluent contamination

Reuse of water is important

- It reduces water cost by 50%
- Effluent treatment cost by 10 fold

Oxygen

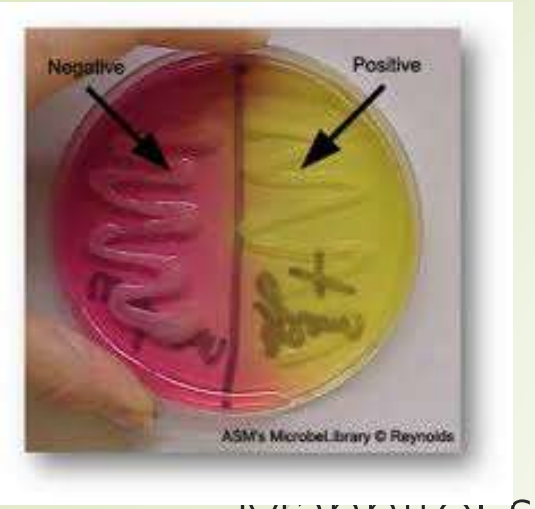
- Depending on the amount of oxygen required by the organism, it may be supplied in the form of air containing about 21% (v/v) oxygen or occasionally as pure oxygen when requirements are particularly high.
- The organism's oxygen requirements may vary widely depending upon the carbon source. For most fermentations the air or oxygen supply is filter sterilized prior to being injected into the fermenter.

Antifoams

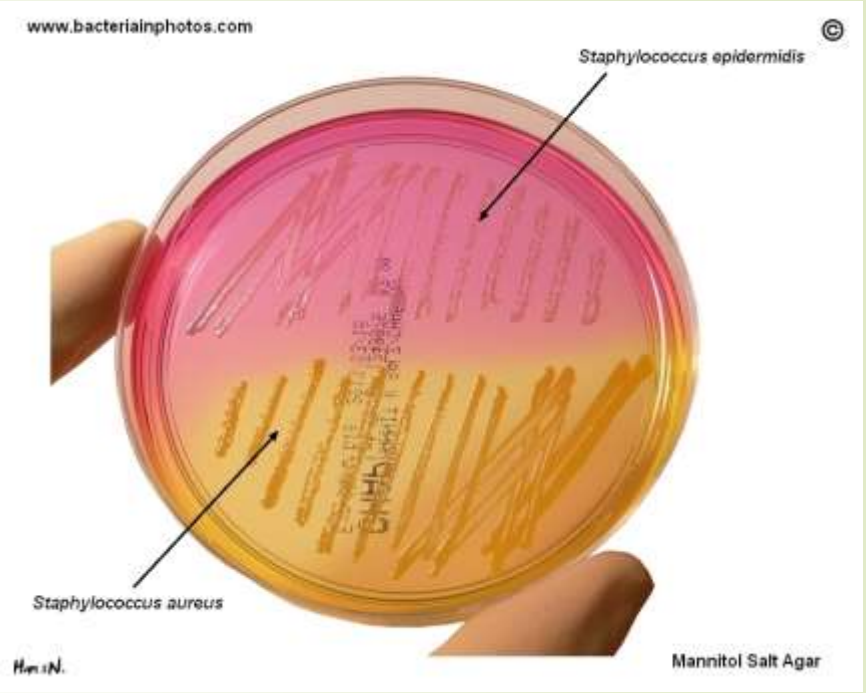
- Antifoams are necessary to reduce foam formation during fermentation.
- Foaming is largely due to media proteins that become attached to the air-broth interface where they denature to form a stable foam “skin” that is not easily disrupted
- An ideal antifoam should have the following properties
  - Disperse readily and have fast action
  - Active at low concentrations
  - Long acting in preventing new foam
  - Should not be metabolized
  - Should not be toxic to m.o, humans etc
  - Cheap, should not cause problem in fermentation

Types of media

- **DEFINED VS UNDEFINED MEDIA**
- COMMON BROADLY-DEFINED CULTURE MEDIA
- **Nutrient media**
- **Minimal media**
- **Selective media**
- **Differential media**
- **Transport media**
- **Enriched media**



Mannitol Salt Agar (MSA):



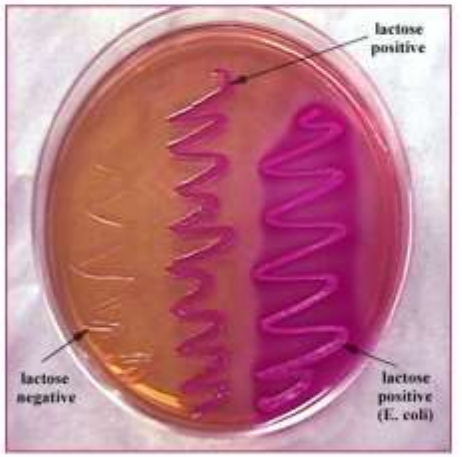
Mannitol salt agar is both a selective and differential media used for the isolation of pathogenic *Staphylococci* from mixed cultures.

Ingredients per liter of deionized water

Beef extract	1.0 g
Peptone	10.0 g
Sodium chloride	75.0 g
D-mannitol	10.0 g
Agar	15.0 g
Phenol red	0.025 g

MacConkey's Agar (MAC):

MacConkey's Agar is both a selective and differential media; it is selective for Gram negative bacteria and can differentiate those bacteria that have the ability to ferment lactose.



Ingredients per liter of deionized water

Bacto peptone	17.0 g
Proteose peptone	3.0 g
Lactose	10.0 g
Bile salts mixture	1.5 g
Sodium Chloride	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g

Composition of Nutrient Agar

- **0.5% Peptone**

It is an enzymatic digest of animal protein. Peptone is the principal source of organic nitrogen for the growing bacteria.

- **0.3% beef extract/yeast extract**

It is the water-soluble substances which aid in bacterial growth, such as vitamins, carbohydrates, organic nitrogen compounds and salts.

- **1.5% agar**

It is the solidifying agent.

- **0.5% NaCl**

The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.

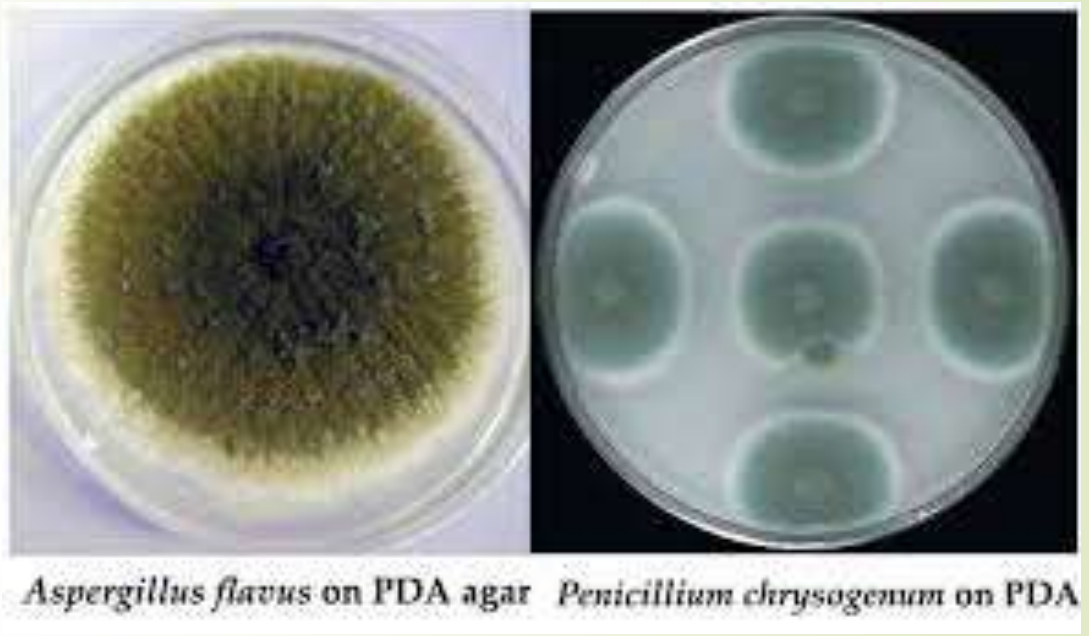
- **Distilled water**

Water is essential for the growth of and reproduction of micro-organisms and also provides the medium through which various nutrients can be transported.

- **pH is adjusted to neutral (7.4) at 25 °C.**

Potato dextrose agar

- Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth.
- It is recommended for plate count methods for foods, dairy products and testing cosmetics. PDA can be used for growing clinically significant yeast and molds.



Aspergillus flavus on PDA agar *Penicillium chrysogenum* on PDA

Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 liter

Medium Optimization

- When considering the biomass growth phase in isolation, it must be recognized that efficiently grown biomass produced by an ‘optimized’ high productivity growth phase is not necessarily best suited for its ultimate purpose, such as synthesizing the desired product.

Classical design

- Changing one variable at time
- Total no of experiments will be x^n
  - x – no of level
  - n - no of variables or factors
  - For ex 3 levels and 6 variables have to be tested then the number of experiments will be $3^6=729$

Plackett Burman design- Statistical optimization technique

- More than five variables it is useful
- It will be useful in screening the most important variable
- Here n no of experiments will be conducted for n-1 variables
- Where n is the multiples of 4 like 8,12,16,20…100
- Authors give a series of experimental design known as balanced incomplete blocks
- Variables which is not having influence in the process is designated as dummy variables
- Dummy variables are required to estimate the error in the experimentation
- Minimum one or two dummy variables should be included in the experimental set
- More can be included if the real variables are less

TABLE 4.16. *Plackett–Burman design for seven variables* (Nelson, 1982)

Trial	Variables							Yield
	A	*B*	*C*	*D*	*E*	*F*	*G*	
1	H	H	H	L	H	L	H	1.1
2	L	H	H	H	L	H	L	6.3
3	L	L	H	H	H	L	H	1.2
4	H	L	L	H	H	H	L	0.8
5	L	H	L	L	H	H	H	6.0
6	H	L	H	L	L	H	H	0.9
7	H	H	L	H	L	L	H	1.1
8	L	L	L	L	L	L	L	1.4

H denotes a high level value; L denotes a low level value.

TABLE 4.17. *Analysis of the yields shown in Table 4.16* (Nelson, 1982)

	Factor						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Σ(H)	3.9	14.5	9.5	9.4	9.1	14.0	9.2
Σ(L)	14.9	4.3	9.3	9.4	9.7	4.8	9.6
Difference	−11.0	10.2	0.2	0.0	−0.6	9.2	−0.4
Effect	−2.75	2.55	0.05	0.00	−0.15	2.30	−0.10
Mean square	15.125	13.005	0.005	0.000	0.045	10.580	0.020

Mean square for 'error' $= \frac{0.045 + 0.020}{2} = 0.0325$

Recent update

Streptomyces flavogriseus HS1: Isolation and Characterization of Extracellular Proteases and Their Compatibility with Laundry Detergents

- ISP4 agar media containing (g/L) starch 10, casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4 \cdot 7H_2O$ 0.01, and 15 agar
- FermII media: (g/L) dextrin 20, tryptone 10, KH_2PO_4 1.0, K_2HPO_4 3.4, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnCl_2$ 0.1, $CuSO_4 \cdot 7H_2O$ 0.01, $MgCl_2 \cdot 4H_2O$ 0.003, $CaCl_2$ 0.01, NaCl 0.03, pH 7.0 [18],
- gelatin containing media: (g/L): gelatin, 10; peptone, 5; yeast extract, 5; NaCl, 50; and pH 9,
- and liquid ISP4 media


Thank
You