17BTU414A INDUSTRIAL FERMENTATION PRACTICAL 3H - 1C
Total hours/week: L:0 T:0 P:3 Marks: Internal: 40 External: 60 Total: 100

Practical

1. Comparative analysis of design of a batch and continuous fermenter.
2. Calculation of Mathematical derivation of growth kinetics.
3. Solvent extraction & analysis of a metabolite from a bacterial culture.
4. Perform an enzyme assay demonstrating its hydrolytic activity (protease/peptidase/glucosidase etc.)

References

1. Stanbury, P.F., Whitaker ,A. & Hall, S.J. (2006). *Principles of Fermentation Technology* (2nd ed.). Elsevier Science Ltd.
2. Crueger, W., & Crueger, A. (2000). *Biotechnology: A textbook of Industrial Microbiology* (2nd ed.). New Delhi: Panima Publishing Co.
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1. Comparative analysis of design of a batch and continuous fermenter.

Aim: To compare the design of Batch and Continous fermenter

Definition:

A Fermentor can be defined as a vessel in which sterile nutrient media and pure culture of micro-organism are mixed and fermentation process is carried out under aseptic and optimum condition. Fermentor provides a sterile environment and optimum condition that are important for growth of micro-organisms and synthesis of desired product.

A fermentor should be constructed in such a way that it can make provisions for the below activities:

1. Sterilization
2. Temperature control
3. pH control
4. Foam control
5. Aeration and agitation
6. Sampling point
7. Inoculation points for micro-organisms, media and supplements
8. Drainage point for drainage of fermented media
9. Harvesting of product
10. Cleaning
11. Facility of providing hot, cold water and sterile compressed air.

Major parts of fermentor and their function.

1. Material used for fermentor

The material used for designing of a fermentor should have some important functions.
It should not be corrosive
It should not add any toxic substances to the fermentation media.
It should tolerate steam sterilization process.
It should be able to tolerate high pressure and resist pH changes.

2. Impellers

Impellers are an agitation device. They are mounted on the shaft and introduced in the fermentor through its lid.
They are made up of impeller blades and the position may vary according to its need.
These impellers or blades are attach to a motor on lid.

The important function of an impeller is to mix micro-organisms, media and oxygen uniformly.
Impeller blades reduce the size of air bubbles and distributes these air bubbles uniformly into the fermentation media.
Impellers also helps in breaking foam bubbles in the head space of fermentor. This foam formed during fermentation process can cause contamination problem and this problem is avoided by the use of impellers.

3. Baffles

Baffles are mounted on the walls of a fermentor.
The important function of baffles is to break the vortex formed during agitation process by the impellers.
If this vortex is not broken, the fermentation media may spill out of fermentor and this may result in contamination as well as can lead to different problems. So it is important to break the vortex formed by using a barrier.
Baffles acts as a barrier which break the vortex.

4. Inoculation port

Inoculation port is a device from which fermentation media, inoculum and substrate are added in the fermentation tank.
Care should be taken that the port provides aseptic transfer.
The inoculation port should be easy to sterilize.

5. Sparger

A Sparger is an aeration system through which sterile air is introduced in the fermentation tank.
Spargers are located at the bottom of the fermentation tank.
Glass wool filters are used in a sparger for sterilization of air and other gases.
The sparger pipes contain small holes of about 5-10 mm. Through these small holes pressurized air is released in the aqueous fermentation media.
The air released is in the form of tiny air bubbles. These air bubbles helps in mixing of media.

6. Sampling point

Sampling point is used for time to time withdrawal of samples to monitor fermentation process and quality control.
This sampling point should provide aseptic withdrawal of sample.

7. pH control device

Sampling point is used for time to time withdrawal of samples to monitor fermentation process and quality control.
This sampling point should provide aseptic withdrawal of sample.

8. Temperature control system

Temperature control device generally contains a thermometer and cooling coils or jackets around fermentor.
During the fermentation process, various reactions take place in the fermentor. Heat is generated and released in the fermentation media. This increase in temperature is detrimental to the growth of micro-organisms, which may slow down the fermentation process.
So, it is necessary to control this rise in temperature. This is done by passing cool water through the coils or jackets present around fermentor.

9. Foam control device

A Foam controlling device is placed on the top of fermentor with a inlet into fermentor. This device contains a small tank containing anti-foaming agent.

Foam is generated during fermentation. It is necessary to remove or neutralize this foam with the help of anti-foaming agents, lest the media may spill out of fermentor and lead into contamination and a mess.

10. Bottom drainage system

It is an aseptic outlet present at the bottom of fermentor for removal of fermented media and products formed.

Continuous Fermentation: Here the exponential growth rate of the microbes is maintained in the fermenter for prolonged periods of time in by the addition of fresh media are regular intervals. The metabolite or the product of fermentation is extracted for the overflow from the fermenter. Thus unlike batch fermentation, in continuous fermentation, the fermentation process never stops in between and it continues to run for a long period of time with the addition of nutrients and harvesting the metabolites at regular intervals.

Batch Fermentation: Here the fermenter is first filled with the raw material (carbon source). Then the microbes are added and allowed to ferment the raw material under optimum pH and aeration. The products remain in the fermenter until the completion of fermentation. After fermentation, the products are extracted and the fermenter is cleaned and sterilized before next round. Thus here the fermentation is done as separate batches.

Basic Design of a Fermenter

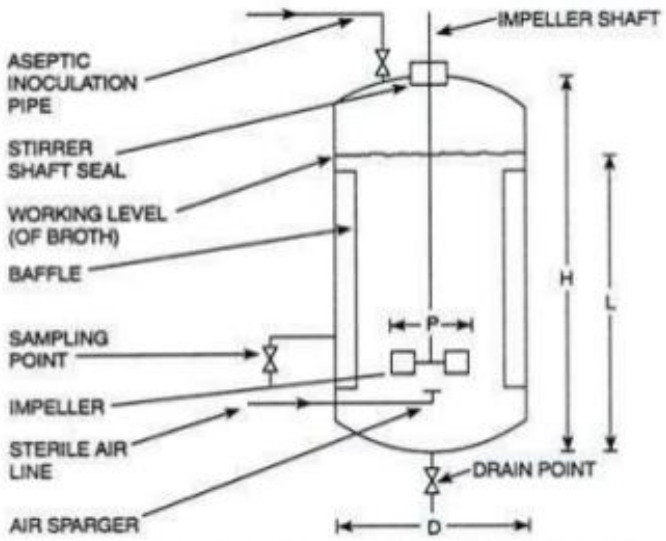


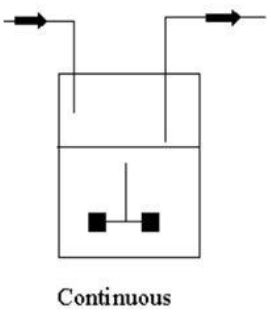
Diagram of a fermenter with one multi-bladed impeller. H, fermenter height; L, liquid height; D, tank diameter; P, impeller diameter.

Various components of an ideal fermenter for batch process are:

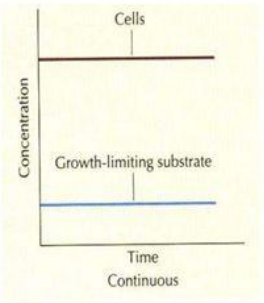
S.No.	Part	Purpose
1	Top plate	cover (made of steel)
2	Clamp	top plate compressed onto vessel using clamp
3	Seal	separates top plate from vessel (glass) to prevent air leakage
4	Vessel	glass, jacketed, steel with ports for various outputs, inputs, probes etc
5	Drive motor	used to drive mixing shaft
6	Drive shaft	mixes the medium evenly with its impeller
7	Marine impeller	for plant tissue culture
8	Baffles	prevent sedimentation on sides and proper mixing
9	Sparger	air supplier / after filtration via membranes – ensures efficient dispersal – by attached to impeller
10	Exit gas cooler	like condenser remove as much moisture as possible from exhaust
11	Inoculation needle	port to add inoculum
12	Feed pumps	regulates the flow rates of additives (medium, nutrients) variable

III. Continuous fermentation

In continuous fermentation, fresh growth medium is added continuously during fermentation, but there is also continuous removal of an equal volume of medium containing suspended microorganisms.



Continuous



(1) A steady-state condition of the total number of cells and the total volume in the bioreactor is maintained.

(2) A single reaction can be maintained for a much longer period.

(3) However, potential drawbacks are loss recombinant plasmid constructs in some cells, difficulty in maintenance of an industrial scale and variation from batch to batch.

Fermentation mode	BATCH	CONTINUOUS
Substrate	1.5 % (w/v) starch	1.0 % (w/v) starch
Additives	0.05 % (w/v) yeast extract	0.05 % (w/v) yeast extract
Whey feed	7.9 % (w/v)	-
Maximum cell density	2.84×10^9 cells/ml	2.7×10^9 cells/ml
Maximum H_2 production rate	**84 ml × l^{-1} × h^{-1}** **3.4 mmol × l^{-1} × h^{-1}**	**147 ml × l^{-1} × h^{-1}** **6.0 mmol × l^{-1} × h^{-1}**
Starch consumption rate	**261 mg × l^{-1} × h^{-1}**	**607 mg × l^{-1} × h^{-1}**
Acetate production rate	**91 mg × l^{-1} × h^{-1}** **1.5 mmol × l^{-1} × h^{-1}**	**198 mg × l^{-1} × h^{-1}** **3.3 mmol × l^{-1} × h^{-1}**
Ethanol production rate	**4 mg × l^{-1} × h^{-1}** **0.08 mmol × l^{-1} × h^{-1}**	**102 mg × l^{-1} × h^{-1}** **2.2 mmol × l^{-1} × h^{-1}**

2.Calculation of Mathematical derivation of growth kinetics.

Aim: To Measure the Kinetics of Microbial Growth

Microbial growth is described as an orderly increase in all chemical components in the presence of suitable medium and the culture environment. There are four types of microbial growth: bacteria grow by binary fission, yeast divide by budding, fungi divide by chain elongation and branching and viruses grow intracellularly in host cells.

MEASUREMENT OF MICROBIAL GROWTH Growth of the cell mass or cell number can be described quantitatively as a doubling of the cell number per unit time for bacteria and yeasts or a doubling of biomass per unit time for filamentous organisms such as fungi. Measuring techniques involve direct counts, visually or using instruments and indirect cell counts. The first method is to measure the dry weight of the cell material in a fixed volume of the culture by measuring the dry weight of the cell material in a given volume of the culture. The cells need to be removed from the medium and dried. Another method is to use the spectrophotometer to estimate absorbance of cell suspensions. The absorbance at a particular wavelength is proportional to the cell concentration. By plotting a standard curve of absorbance versus cell concentration, the cell concentration of an unknown sample can be calculated. Direct microscopic counts using a counting chamber can be used but this technique has limitations as the dead cells cannot be distinguished from living cells. Electronic counting chambers count numbers and measure size distribution of cells. These are more often used to count eucaryotic cells like blood cells. Indirect viable cell counts also called plate counts may be used. This involves plating out dilutions of a culture on nutrient agar. Each viable unit will form a colony and each colony that can be counted is called a colony forming unit (cfu) and the number of cfus is related to the viable count in the sample. Turbidity measurement is a fast and nondestructive method especially for counting large numbers of bacteria in clear liquid media and broths – but cannot detect cell densities less than 107 cells per ml. The biochemical activity may also be measured – e.g., O2 uptake, CO2 production, ATP production. This method requires a fixed standard to relate chemical activity to cell mass and/or volume. Bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH etc.) define the bacterium's generation time. Generation times for bacteria vary from about 12 minutes to 24 hours. The generation time for E. coli in the laboratory is 15-20 min. Symbionts such as Rhizobium tend to have a longer generation time. Some pathogenic bacteria, e.g., Mycobacterium tuberculosis have especially long generation times and this is thought to be an advantage to their virulence. When growing exponentially by binary fission, the

increase in a bacterial population is by geometric progression. The generation time is the time interval required for cells (or population) to divide:

G =t/n

Where G is generation time, n is number of generations and t is time in min/hours

The equation for growth by binary fission is: b = B x 2n

where b is number of bacteria at end of a time interval, B is number of bacteria at beginning of a time interval, n is the number of generations (number of times the population doubles in the time interval).

logb = logB + nlog2

$$\log b = \log B + n \log 2$$

$$n = \frac{\log b - \log B}{\log 2}$$

$$n = \frac{\log b - \log B}{.301}$$

$$n = 3.3 \log b/B$$

$$G = \frac{t}{3.3 \log b/B}$$

GROWTH KINETICS IN BATCH CULTURE Batch culture occurs in a closed system that contains an initial limited amount of substrate. The inoculated microorganism will pass through a number of growth phases. During the log phase, cell numbers increase exponentially at a constant maximum rate. In mathematical terms, we can write:

$$\frac{dx}{dt} = \mu x$$

where x is the concentration of microbial biomass, t is the time in hours and μ is the specific growth rate in hours-1. If we integrate between time t0 and time t1 when the concentrations of the cells are X0 and X1 we obtain:

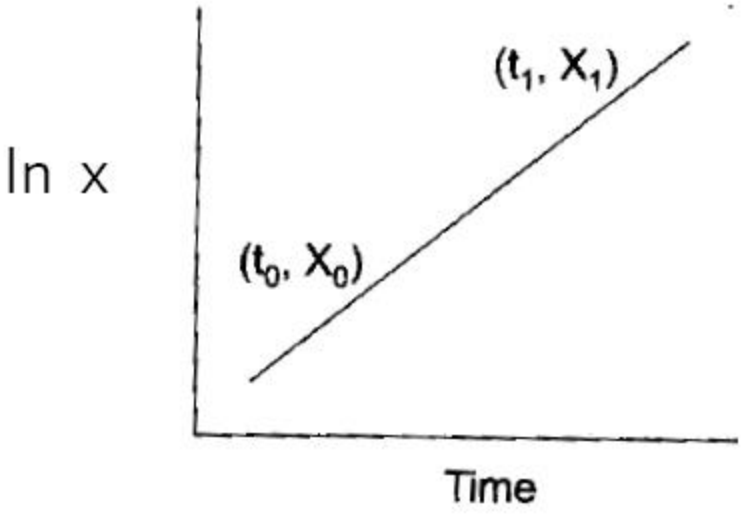
$$x_t = x_o e^{\mu t}$$

where x0 is the original biomass concentration, xt is the biomass concentration after a time interval t hours and e is the base of the natural logarithm. On taking natural logarithms, the equation becomes:

$$\ln x_t = \ln x_o + \mu t$$

Using this equation, a plot of the natural log of biomass concentration versus time should yield a straight line, the slope of which will equal the specific growth rate (μ).

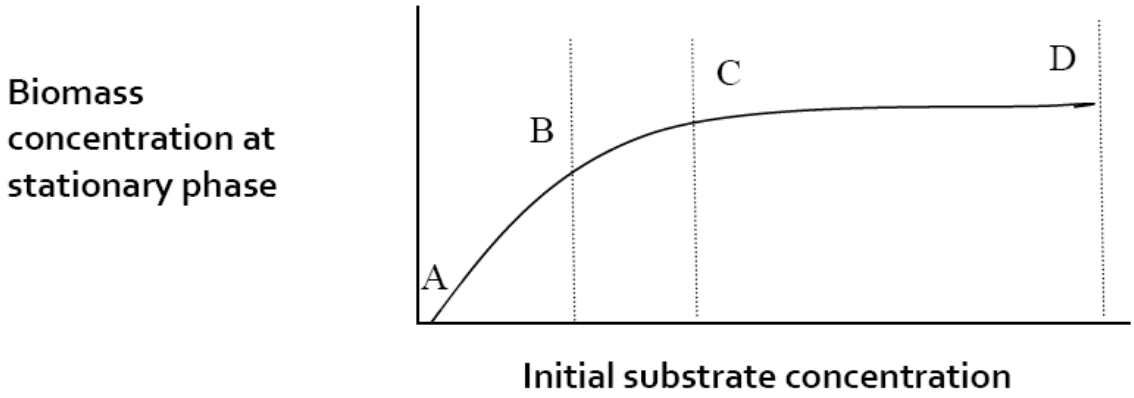
$$\mu = \text{slope} = \frac{\ln (X_1 - X_0)}{t_1 - t_1}$$



During the exponential phase, nutrients are in excess and the microorganism is growing at maximum specific growth rate μmax for the prevailing conditions.

The major problem of the exponential growth equation is that it does not predict an end to growth in a batch environment. According to this model, not only the whole earth, but also the whole solar system could become quickly covered by bacteria. However, growth results in the consumption of nutrients and the excretion of microbial products. After a time, the growth rate of the culture ceases. The cessation of growth may be due to the depletion of some essential nutrient in the medium (substrate limitation), the accumulation of some autotoxic product in the medium or a combination of the two.

The nature of the limitation of growth can be explored by growing the microorganisms in a range of substrate concentrations and plotting the biomass concentration in the stationary phase against the initial substrate concentration, the nature of growth limitation may be explored.


Biomass concentration at stationary phase
A
B
C
D
Initial substrate concentration

The effect of initial substrate concentration on the biomass concentration at the onset of stationary phase in batch culture

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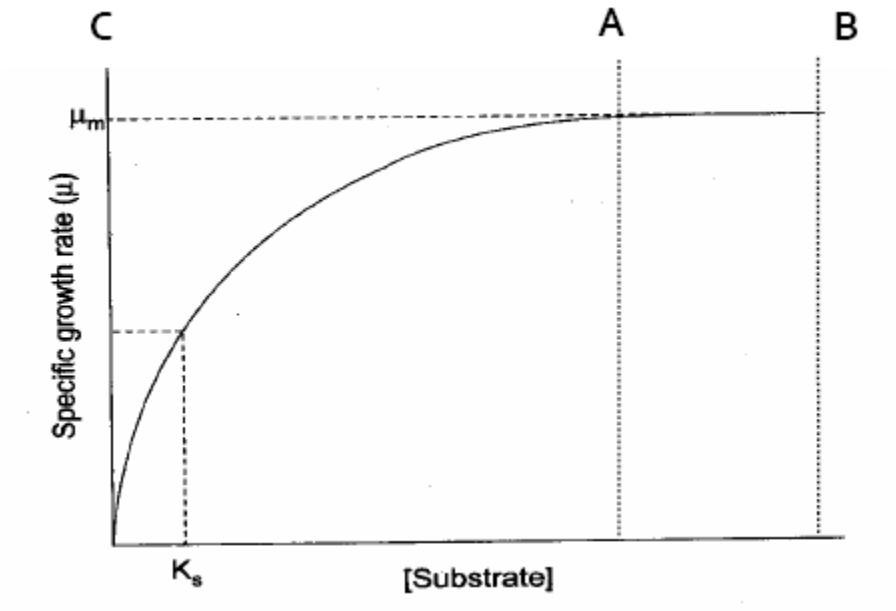
$$x = Y\,(S_R - S)$$

Where x is the concentration of biomass produced, Y is the yield factor (g biomass produced per g substrate utilized), SR is the initial substrate concentration and S is the residual substrate concentration. In the area between A and B, S is zero and therefore the equation above could be used to predict the biomass that could be formed from a certain amount of substrate. Between B and C although biomass increases with increasing substrate concentration, there is a diminished effect due to accumulation of toxic products or reduced availability of some other substrate. In the region between C and D, there is no change in biomass with increasing substrate concentration which may be attributed to increasing levels of toxic products or the exhaustion of some other substrate.

Y, the yield factor is the measure of efficiency of conversion of any one substrate to biomass. Although Y is not a constant and varies according to growth rate, pH, temperature, the limiting substrate and concentration of the substrate in excess, it can be used to predict the substrate concentration required to produce a certain biomass concentration. In the 1930s, Jacques Monod performed a number of initial rate experiments and plotted the specific growth rate against the concentration of growth-limiting substrate (Fig. 2.5). The result was a Langmuir type graph that appeared similar to enzymatic rate-substrate relationships described by Michaelis-Menton's model. Monod's model describing the relationship between the specific growth rate and the growth limiting substrate concentration is:



$$\mu = \frac{\mu_m S}{K_S + S}$$

The effect of residual limiting substrate concentration on specific growth rate of a hypothetical bacterium.

Where μm is the maximum specific growth rate, S is the residual substrate concentration and Ks is the substrate utilization constant, numerically equal to substrate concentration when μ is half μm μm and is a measure of the affinity of the organism for its substrate. Zone A to B in Fig. represents the exponential phase of growth in batch culture where substrate concentration is in excess and growth is at μm. Zone A to C is the deceleration phase, substrate concentration becomes limiting and cannot support growth at μm. An organism with a high affinity for the limiting substrate (low Ks) will have a short deceleration phase as the growth rate will only be affected when the substrate concentration is very low. Conversely, a microorganism with a low affinity for the substrate will have a very long deceleration phase (growth slows down at high substrate concentrations). The point when growth rate has declined to zero represents the stationary phase. This is a misnomer as many organisms are still metabolically active and are producing products called secondary metabolites during this phase.

Monod's model is widely used to describe the growth of many microorganisms. The equation adequately describes fermentation kinetics and can be used to describe complex fermentation systems. The equation adequately describes fermentation kinetics and can be used to describe complex fermentation systems, e.g., a commonly used expression to describe product inhibition is:

$$\mu = \left[1 - \frac{P}{P_m}\right] \frac{\mu_m S}{K_S + S}$$

Using the Monod model, a simple model microbial growth can be written as:

$$\frac{dX}{dt} = \mu X = \frac{\mu_m S}{K_S + S} X$$

$$\frac{dS}{dt} = -\frac{1}{Y_{xs}} \mu X = -\frac{1}{Y_{xs}} \frac{\mu_m S}{K_S + S} X$$

where Yxs is the biomass yield coefficient. The biomass yield coefficient is the efficiency of conversion of substrate to biomass and is calculated as:

$$\text{Biomass} = \frac{\text{Dry weight of biomass produced}}{\text{Weight of substrate used}}$$

The kinetics of product formation may be described as growth-linked products and non-growth linked products. In the first instance – these could relate to primary metabolites synthesized by growing cells and the non-growth-linked products would be secondary metabolites. Formation of growth-linked products can be defined by the following:

$dp/dt = qpx$

where p is the concentration of product, qp is the specific rate of product formation (mg product /g biomass/h).

Product formation can also be expressed in relation to biomass as:

$dp/dx = Yp/x$

where Yp/x is the yield of product in terms of biomass (g product/g biomass). Combining these equations:

$qp = Yp/x \cdot \mu$

Thus when product formation is linked to growth, the specific rate of product formation increases with specific growth rate and will be highest at μm. In this instance improved output will be obtained by increasing both biomass and μ. Non-growth linked product formation is related to biomass concentration. As these products are produced only under certain physiological conditions (usually limitation of a certain substrate), the biomass needs to be in the correct physiological state before secondary metabolites are produced.

Batch fermentations may be used to produce biomass and primary and secondary metabolites. For (i) biomass production: conditions supporting fastest growth rate and maximum cell concentration;

For (ii) primary metabolites: conditions to extend exponential phase accompanied by product excretion;

For (iii) secondary metabolites: conditions providing a short exponential phase and extended production phase or conditions giving decreased growth rate in the log phase resulting in earlier secondary metabolite production.

3. Solvent extraction & analysis of a metabolite from a bacterial culture.

Aim: To follow liquid-liquid extraction method for extraction of metabolite from bacterial culture.

Principle:

In general, a solute dissolves best in a solvent that is most similar in chemical structure to itself. The overall solvation capacity of a solvent depends primarily on its polarity. For example, a very polar solute such as urea is very soluble in highly polar water, less soluble in fairly polar methanol, and almost insoluble in non-polar solvents such as chloroform and ether. Nucleic acids are polar because of their negatively charged phosphate backbone, and therefore nucleic acids are soluble in the upper aqueous phase instead of the lower organic phase (water is more polar than phenol). Conversely, proteins contain varying proportions of charged and uncharged domains, producing hydrophobic and hydrophilic regions. In the presence of phenol, the hydrophobic cores interact with phenol, causing precipitation of proteins and polymers (including carbohydrates) to collect at the interface between the two phases (often as a white flocculent) or for lipids to dissolve in the lower organic phase.

Materials and Methods:

Materials Reagents • TRIzol reagent • Chloroform, a fume hood, vortexer, micropipettes, chilled microcentrifuge, pellet pestle homogenizer, and all standard equipment of a biochemistry-molecular biology laboratory.

Procedure:

Homogenization Bacterial cultured cells. Procedure is done at room temperature (15-25 ºC) unless otherwise indicated. Cell cultures should be processed immediately after removal from the incubator. Either centrifuge cells grown in suspension at 300 x g for 5 min at RT (15-25 ºC) and discard supernatant or remove the culture medium from cells grown in monolayer. In both cases, it is not necessary to wash the cells with saline. Add 1 ml TRIzol reagent per 1 × 107 cells to cell pellets or directly to the culture dish or flask for cells grown in monolayer. Resuspend the lysate with a sterile, disposable 1-ml pipette tip

Extraction Transfer the cell lysate to a 2-ml siliconized low-retention tube. Pass sample through sterile, disposable 21 g needle 10 times. In doing so, you will fragment high-molecular weight cellular components (DNA), thus minimizing their presence in the aqueous phase. | Let homogenate sit at RT (15-25 ºC) for 5 min for complete dissociation of nucleoprotein complexes. You can stop at this point, store your samples at –20 °C, and complete the procedure later. Add 200 ul chloroform. Vortex vigorously for 15 s. Thorough mixing is important for subsequent phase separation. When mixing and shaking, make sure that the caps are tightly closed. Let

homogenate sit at RT (15-25 ºC) for 3 min. Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C). Transfer upper aqueous phase (600 ul) to new 1.5 ml RNase-free tube. Volume of lysate may be less than 600 ul due to loss during homogenization and centrifugation. Proceed to either silica-gel membrane purification or alcohol precipitation.

Result:

Liquid-liquid extractions used to isolate crude proteins or secondary metabolites from the bacterial cell.