THE NEOTIA UNIVERSITY

BIOPROCESS ENGINEERING & TECHNOLOGY

Practical Manual

Course Code: CC-BTLP901

2020

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1. **Experiment No.: CC-BTLP901/01**
2. **Experiment Name:** Exit age distribution curve 'E' for liquid flowing through a vessel (RTD)
3. **Objectives:** To determine exit age distribution curve 'E' for liquid flowing through Continuous Stirred Tank Reactor (CSTR).
4. **Principle:**

   A well-mixed tank is an analogue for many control volumes used in environmental engineering. For example, in our lake example it might be reasonable to assume that pollutants dumped into the lake are rapidly mixed throughout the entire lake. In environmental engineering and chemical engineering, the term *Continuously Stirred Tank Reactor* or *CSTR* is used for such a system. An example of a CSTR is shown in Figure 1.

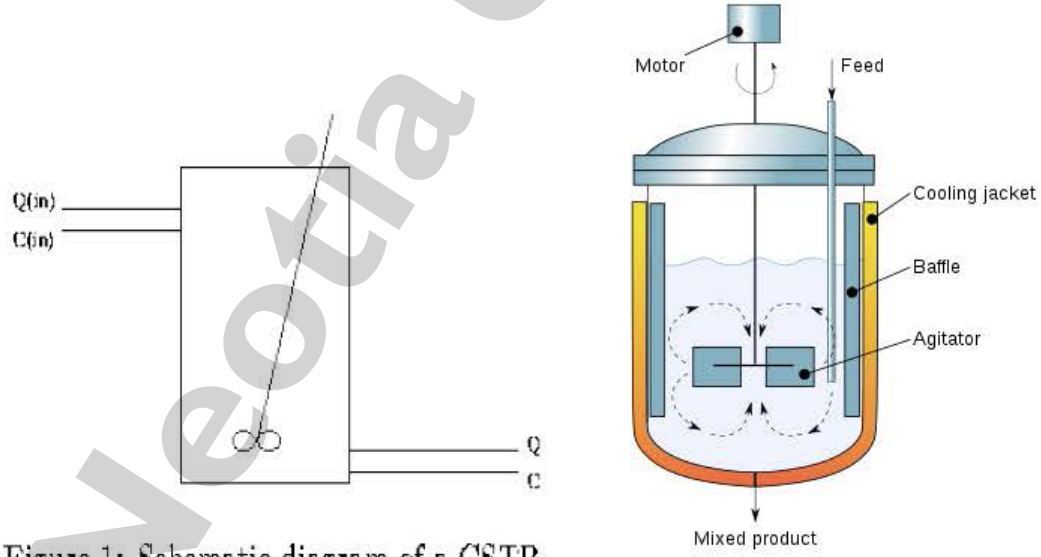
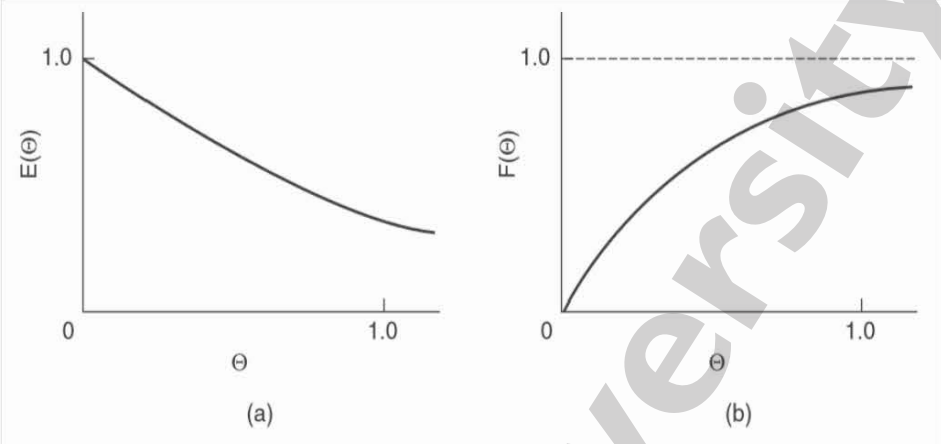


Figure 1: Schematic diagram of a CSTR

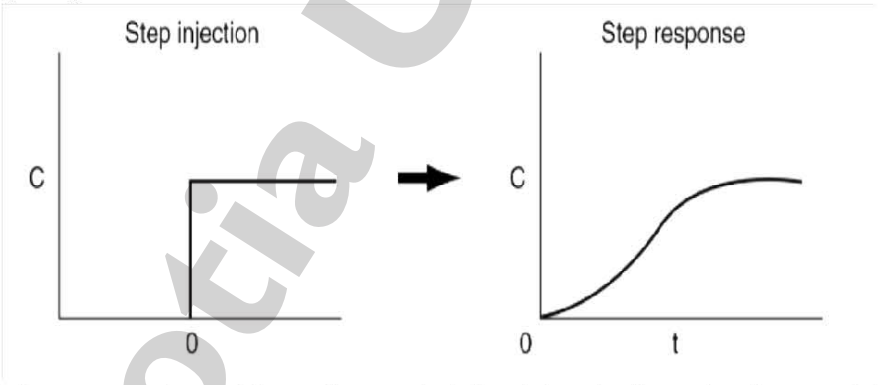
The Residence Time Distribution (RTD) inside the Continuous Stirred Tank Reactor (CSTR) shows the characteristic of the mixing of reactants that occurs inside the reactor. Inside the reactor, the reactants are continually consumed as the reactant flow along the length of the reactor. The residence time distribution function is represent in a plotted graph of E(t) as a function of time. This function shows in a quantitative manner of how much time the mixed fluid stays inside the reactor before leaving the reactor.



(a) E(t) curve and (b) F(t) Curve For Ideal CSTR

RTD is determined experimentally by injecting chemically inert substance known as tracer into the reactor at t = 0 and measuring the concentration of the tracer at the effluent stream as a function of time. The two most common methods of injecting tracer into the reactor are pulse input and step input.

Step Change Input In Continuous Stirred Tank Reactor



Typical Concentration – Time Curve At The Inlet And Outlet Stream For Step Change Input

A constant rate of tracer is added to the feed that is initiated at time t = 0. Thus, the inlet concentration of the tracer, C_o is constant with time. From this experiment, the cumulative distribution can be determined directly, F(t).

$$C_o(t) = \begin{cases} 0 & t < 0 \\ (C_o)\ \text{constant} & t \leq 0 \end{cases}$$

The cumulative distribution, F(t) represents the fraction of effluent that has been in reactor for time t = 0 until t = t.

$$\text{Cumulative Distribution, } F(t) = \left[\frac{C_{out}}{C_0}\right]_{step}$$

Differentiation of the cumulative distribution function yield to RTD function,

$$\text{Residence Time Distribution Function, } E(t) = \frac{d}{dt}\left[\frac{C(t)}{C_0}\right]_{step}$$

The mean residence time, tm shows the average time the fluids stay inside the reactor

$$\text{First Moment, Mean Residence Time, } t_m = \int_0^\infty t\,E(t)\,dt$$

The spread of the distribution which is the magnitude of the variance, σ^2. The greater the magnitude, the greater the distribution's spread will be

$$\text{Second Moment, Variance, } \sigma^2 = \int_0^\infty (t - t_m)^2 E(t)\,dt$$

The extent that a distribution is skewed in one direction is measured by the sleekness's magnitude which also means how differs the distribution is compared to the normal distribution

$$\text{Third Moment, Skewness, } s^3 = \frac{1}{\sigma^{\frac{3}{2}}}\int_0^\infty (t - t_m)^3 E(t)\,dt$$

5. Materials

a) Deionized Water.

b) Oxalic acid

c) Sodium Hydroxide (NaOH)

d) Sulfuric acid (H_2SO_4)

e) Sodium Chloride, NaCl.

f) Phenolphthalein Indicator

6. Procedure:

a) Standardized solution of 0.05(N) oxalic acid is used for back titration.

b) **General Start-Up Procedures**

i) The 20-L feed tank is filled with deionized water.

ii) Take 20 ml of 1(N) H_2SO_4 in a syringe and inject all of it at one instant in the reactor and start the stopwatch simultaneously.

(Sometimes add tracer chloride is dissolved in tank. The salt is made sure to dissolve completely and the solution is ensure to be homogenous).

iii) The power for the control panel is turned on.

iv) The stirrer assembly is made sure to secure properly to avoid damage to the mechanical seal.

v) The way valve is set to position, so that the deionised water from tank will flow into reactor.

vi) Pump is switched on to fill up the reactors with deionised water.

vii) After 5 minutes, pump is then switched off. The way valve is set to position.

vii) The needle valve is adjusted as to set the flow rate, flow rate to 150mL/min. Stirrers is then switched on at ~180 rpm.

c) Start the flow of water in the CSTR and maintain it at a constant value also start the stirrer and keep the speed of agitation sufficient enough for proper mixing and at a constant value.

d) Take 20 ml of solution in a syringe and inject all of it at one instant in the reactor and start the stopwatch simultaneously.

e) Take known volume of sample at the outlet of the reactor every ten seconds and titrate this using 0.05N NaOH

f) Take at least 10 readings.

7. Observations:

a) Normality of NaOH = 0.05N

b) Flow rate of water = F_w (ml/sec)

c) Normality of Oxalic Acid = 0.05N

d) Mean residence time in the reactor = t = V/ Fw (sec)

7.1 Observation Table:

Sl No	Volume of sample V_2 (ml	Volume of NaOH (ml)	Outlet concentration of H_2SO_4 C(t)	Time (t) seconds	E(t)	t E(t)	$(t-tm)^2$ E(t)

7.2 Calculations:

Plot concentration of NaOH (Ca) versus time and determine the area under the curve

Find $E(t) = C(t) / \int C(t)\, dt$

t_m = Mean residence time = $\int t.\, E(t)\, dt$ (min)

σ^2 (Variance) = $\int (t-tm)^2 . t .dt$ $(min)^2$

σ_θ^2 (skewness) = σ^2 / t_m^2 (dimensionless)

$\sigma_\theta^2 = 2/Pe - 2/Pe^2 (1-e^{-Pe})$

By trial & error

Pe = ----------

7.3 Graphs:

Plot the graph of C(t) v/s time

Plot the graph of E(t) v/s time

Plot the graph of t E(t) v/s time

Plot the graph of $(t-tm)^2$ E(t) v/s time

8. Inference/discussion

9. Safety precautions

General Shut-Down Procedures

i) The pumps and stirrer is switched off. The needle valve is closed.

ii) All liquids in the reactors are drained by opening valves.

iii) The power for the control panel is switched off.

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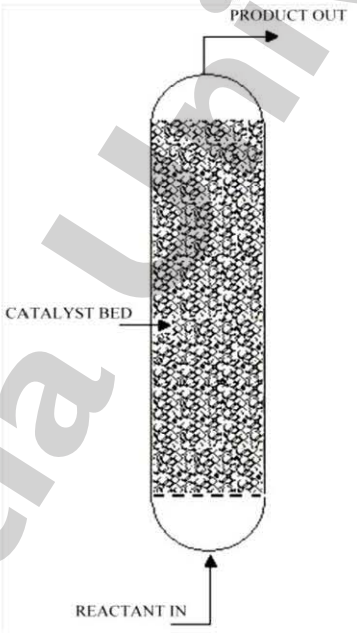
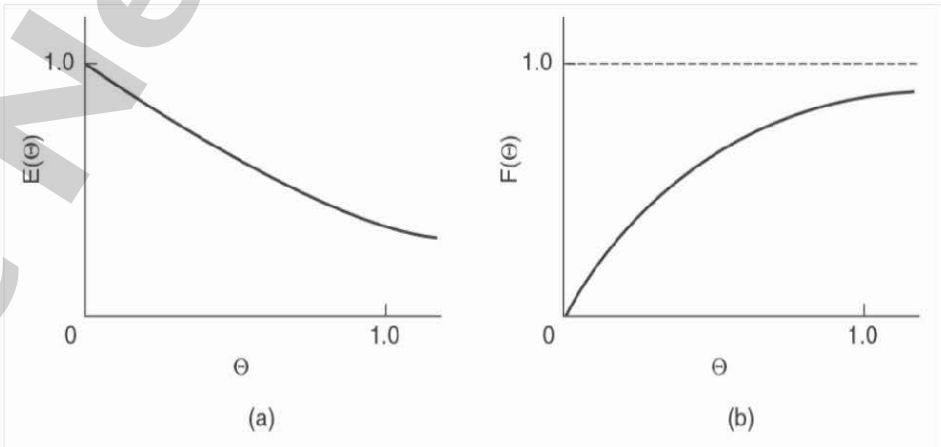
1. **Experiment No.: CC-BTLP901/02**
2. **Experiment Name:** Transforming an experimental C_{pulse} curve in to an E curve.
3. **Objective:** To determine an experimental C_{pulse} curve in to an E curve for liquid flowing through packed bed reactor (PBR).
4. **Principle:**

The Residence Time Distribution (RTD) inside the Packed Bed Reactor (PBR) shows the characteristic of the mixing of reactants that occurs inside the reactor. Inside the reactor, the reactants are continually consumed as the reactant flow along the length of the reactor. The residence time distribution function is represent in a plotted graph of E(t) as a function of time. This function shows in a quantitative manner of how much time the mixed fluid stays inside the reactor before leaving the reactor.



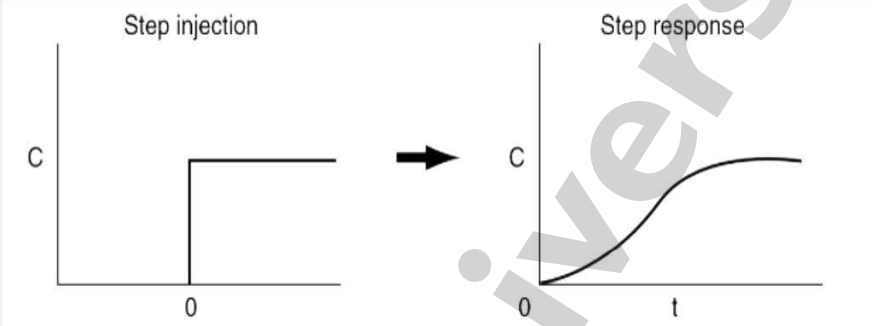
Packed Bed Reactor



(b) E(t) curve and (b) F(t) Curve For Ideal CSTR

RTD is determined experimentally by injecting chemically inert substance known as tracer into the reactor at t = 0 and measuring the concentration of the tracer at the effluent stream as a function of time. The two most common methods of injecting tracer into the reactor are pulse input and step input.

Step Change Input In Continuous Stirred Tank Reactor



Typical Concentration – Time Curve At The Inlet And Outlet Stream For Step Change Input

A constant rate of tracer is added to the feed that is initiated at time t = 0. Thus, the inlet concentration of the tracer, C_o is constant with time. From this experiment, the cumulative distribution can be determined directly, F(t).

$$C_o(t) = \begin{cases} 0 & t < 0 \\ (C_o)\ \text{constant} & t \leq 0 \end{cases}$$

The cumulative distribution, F(t) represents the fraction of effluent that has been in reactor for time t = 0 until t = t.

$$\text{Cumulative Distribution, } F(t) = \left[\frac{C_{out}}{C_0}\right]_{step}$$

Differentiation of the cumulative distribution function yield to RTD function,

$$\text{Residence Time Distribution Function, } E(t) = \frac{d}{dt}\left[\frac{C(t)}{C_0}\right]_{step}$$

The mean residence time, tm shows the average time the fluids stay inside the reactor

$$\text{First Moment, Mean Residence Time, } t_m = \int_0^\infty t\,E(t)\,dt$$

The spread of the distribution which is the magnitude of the variance, σ^2. The greater the magnitude, the greater the distribution's spread will be

$$\text{Second Moment, Variance, } \sigma^2 = \int_0^\infty (t - t_m)^2 E(t)\,dt$$

The extent that a distribution is skewed in one direction is measured by the sleekness's magnitude which also means how differs the distribution is compared to the normal distribution

$$\text{Third Moment, Skewness, } s^3 = \frac{1}{\sigma^3} \int_0^\infty (t - t_m)^3 E(t)\, dt$$

5. **Materials**
   a) Deionized Water
   b) Oxalic acid
   c) Sodium Hydroxide (NaOH)
   d) Sulfuric acid (H_2SO_4)
   e) Sodium Chloride, NaCl
   f) Phenolphthalein Indicator

6. **Procedure:**
   a) Standardized solution of 0.05(N) oxalic acid is used for back titration.
   b) **General Start-Up Procedures**

      i) The 20-L feed tank is filled with deionized water.

      ii) Take 20 ml of 1(N) H_2SO_4 in a syringe and inject all of it at one instant in the reactor and start the stopwatch simultaneously.

      (Sometimes add tracer chloride is dissolved in tank. The salt is made sure to dissolve completely and the solution is ensure to be homogenous).

      iii) The power for the control panel is turned on.

      iv) The stirrer assembly is made sure to secure properly to avoid damage to the mechanical seal.

      v) The way valve is set to position, so that the deionised water from tank will flow into reactor.

      vi) Pump is switched on to fill up the reactors with deionised water.

      vii) After 5 minutes, pump is then switched off. The way valve is set to position.

      vii) The needle valve is adjusted as to set the flow rate, flow rate to 150mL/min. Stirrers is then switched on at ~180 rpm.

   c) Start the flow of water in the Packed bed reactor to build up the liquid level and to achieve steady state.
   d) Measure the steady state flow rate.
   e) Withdraw the sample at regular interval of time and titrate it against std. 0.05N oxalic acid.
   f) Take 18 to 20 such readings.

g) Plot graph of C(t) Vs time, E(t) Vs time , t.E(t) Vs time and (t^2). E(t) vs time using the readings of observation table and calculate area under the curve of each graph.

6. Observations:

1) Normality of Oxalic acid = 0.05N

2) Flow rate = _____ml/sec

3) Normality of NaOH = 0.05N

6.1 Observation Table:

Sl no	Volume of sample V_2 (ml	Volume of NaOH (ml)	Outlet concentration of H_2SO_4C(t)	Time (t) seconds	E(t)	t E(t)	$(t-tm)^2$ E(t)

6.2 Calculations:

From graph calculate the values of

$\int C(t)\, dt$ = _____

t_m = Mean residence time =

$\int t.\, E(t)\, dt$ (min) 0

$\sigma^2 = \int (t-tm)^2 .E(t) .dt\ (min)^2$

$\sigma_\theta^2 = \sigma^2 / t_m^2$ (dimensionless)

$\sigma_\theta^2 = 2/Pe - 2/Pe^2 (1 - e^{-Pe})$

Hence calculate peclet no (by trial and error) from above equation.

7. **Inference/discussion**

8. **Safety precautions**

General Shut-Down Procedures

i) The pumps and stirrer is switched off. The needle valve is closed.

ii) All liquids in the reactors are drained by opening valves.

iii) The power for the control panel is switched off.

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1. **Experiment No.: CC-BTLP901/03**
2. **Experiment Name:** To determine the relation between F and E curves.
3. **Objective:** To determine the relation between F and E curves for liquid flowing through Plug Flow Reactor (PFR).
4. **Principle:**

The Plug Flow Reactor (PFR) is used to model the chemical transformation of compounds as they are transported in ``pipes.'' The ``pipe'' may represent a river, a region between two mountain ranges through which air flows, or a variety of other conduits through which liquids or gases flow. Of course, it can even represent a pipe. As fluid flows down the PFR, the fluid is mixed in the radial direction, but mixing does not occur in the axial direction---each plug of fluid is considered a separate entity as it flows down the pipe. However, as the plug of fluid flows downstream, time passes. Therefore, there is implicit time dependence even in steady-state PFR problems. However, because the velocity of the fluid in the PFR is constant, time and downstream distance are interchangeable: $t = x/v$. We will use this observation together with the mass balance formulations we have worked with already to determine how pollutant concentrations vary during flow down a PFR.

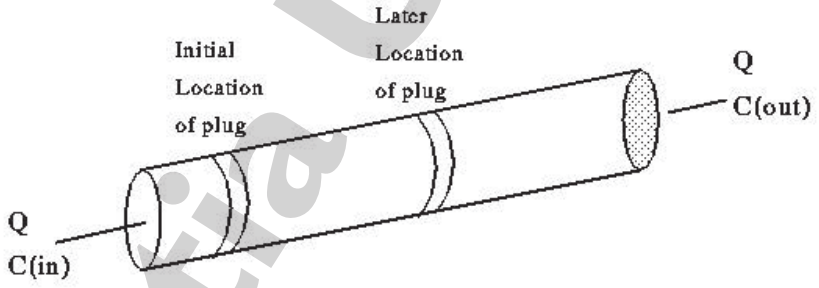
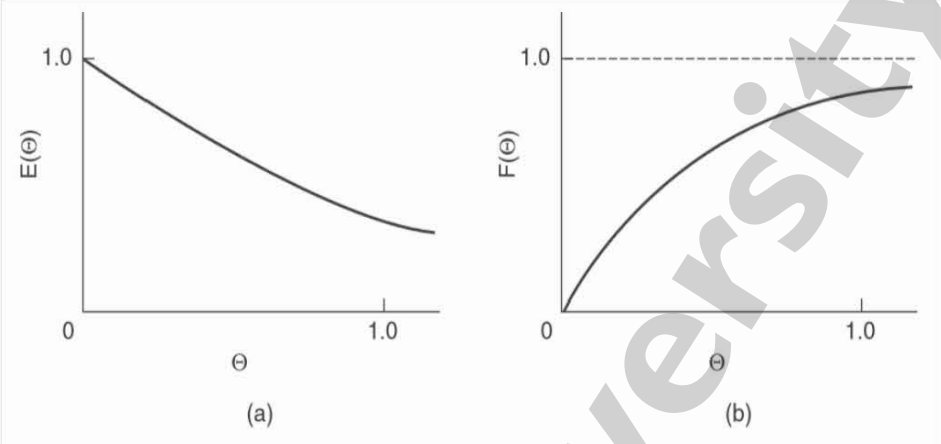


Figure 3: The Plug Flow Reactor

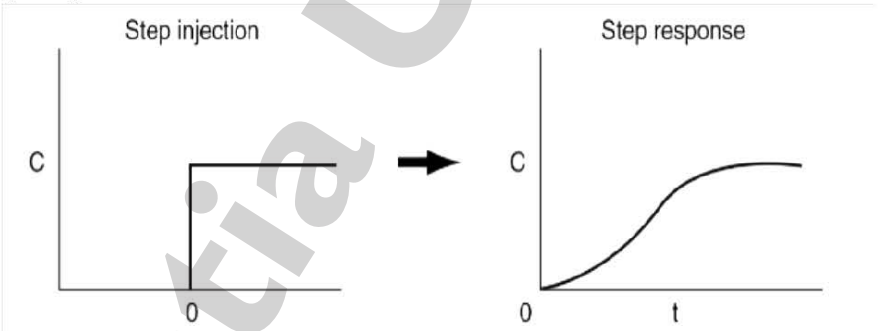
The Residence Time Distribution (RTD) inside the Plug Flow Reactor (PFR) shows the characteristic of the mixing of reactants that occurs inside the reactor. Inside the reactor, the reactants are continually consumed as the reactant flow along the length of the reactor. The residence time distribution function is represent in a plotted graph of E(t) as a function of time. This function shows in a quantitative manner of how much time the mixed fluid stays inside the reactor before leaving the reactor.



(c) E(t) curve and (b) F(t) Curve For Ideal CSTR

RTD is determined experimentally by injecting chemically inert substance known as tracer into the reactor at t = 0 and measuring the concentration of the tracer at the effluent stream as a function of time. The two most common methods of injecting tracer into the reactor are pulse input and step input.

Step Change Input In Continuous Stirred Tank Reactor



Typical Concentration – Time Curve At The Inlet And Outlet Stream For Step Change Input

A constant rate of tracer is added to the feed that is initiated at time t = 0. Thus, the inlet concentration of the tracer, C_o is constant with time. From this experiment, the cumulative distribution can be determined directly, F(t).

$$C_o(t) = \begin{cases} 0 & t < 0 \\ (C_o)\ \text{constant} & t \leq 0 \end{cases}$$

The cumulative distribution, F(t) represents the fraction of effluent that has been in reactor for time t = 0 until t = t.

$$\text{Cumulative Distribution, } F(t) = \left[\frac{C_{out}}{C_0}\right]_{step}$$

Differentiation of the cumulative distribution function yield to RTD function,

$$\text{Residence Time Distribution Function, } E(t) = \frac{d}{dt}\left[\frac{C(t)}{C_0}\right]_{step}$$

The mean residence time, tm shows the average time the fluids stay inside the reactor

$$\text{First Moment, Mean Residence Time, } t_m = \int_0^\infty t\,E(t)\,dt$$

The spread of the distribution which is the magnitude of the variance, σ^2. The greater the magnitude, the greater the distribution's spread will be

$$\text{Second Moment, Variance, } \sigma^2 = \int_0^\infty (t - t_m)^2 E(t)\,dt$$

The extent that a distribution is skewed in one direction is measured by the sleekness's magnitude which also means how differs the distribution is compared to the normal distribution

$$\text{Third Moment, Skewness, } s^3 = \frac{1}{\sigma^{\frac{3}{2}}}\int_0^\infty (t - t_m)^3 E(t)\,dt$$

5. **Materials**
   a) Deionized Water
   b) Oxalic acid
   c) Sodium Hydroxide (NaOH)
   d) Sulfuric acid (H_2SO_4)
   e) Sodium Chloride, NaCl
   f) Phenolphthalein Indicator

6. **Procedure:**
   a) Standardized solution of 0.05(N) oxalic acid is used for back titration.
   b) **General Start-Up Procedures**

      i) The two 20L feed tanks (T1 and T2) are filled with deionized water.

      ii) Take 20 ml of 1(N) H_2SO_4 in a syringe and inject all of it at one instant in the tank 1. The acid is made sure to dissolve completely and the solution is ensuring to be homogenous.

      (Sometimes add tracer chloride is dissolved in tank. The salt is made sure to dissolve completely and the solution is ensure to be homogenous).

      iii) The power for the control panel is turned on.

iv) The stirrer assembly is made sure to secure properly to avoid damage to the mechanical seal.

v) The way valve 1 & 2 are set to position, so that the deionised water and acid solution from tank 1 & 2 will flow into axial pipe of the reactor.

vi) Pump is switched on to fill up the reactors with deionised water.

vii) After 5 minutes, pump is then switched off. The way valve is set to position.

vii) The needle valve is adjusted as to set the flow rate, flow rate to 150mL/min.

c) Start the flow of water in the Packed bed reactor to build up the liquid level and to achieve steady state.
d) Measure the steady state flow rate.
e) Withdraw the sample at regular interval of time and titrate it against std. 0.05N oxalic acid.
f) Take 18 to 20 such readings.
g) Plot graph of C(t) Vs time, E(t) Vs time , t.E(t) Vs time and (t^2). E(t) vs time using the readings of observation table and calculate area under the curve of each graph.

7. **Observations:**

1) Normality of NaOH = 0.05N

2) Flow rate of water = (ml/sec)

3) Normality of Oxalic Acid = 0.05N

4) Mean residense time in the reactor = t = V/ Fw (sec)

7.1 Observation Table:

Sl no	**Volume of sample V_2 (ml**	**Volume of NaOH (ml)**	**Outlet concentration of H_2SO_4C(t)**	**Time (t) seconds**	**E(t)**	**t E(t)**	**$(t-tm)^2$ E(t)**

7.2 Calculations:

Plot concentration of H_2SO_4 (C_a) versus time and determine the area under the curve

Find $E(t) = C(t) / \int C(t)\, dt$

t_m = Mean residence time = $\int t.\, E(t)\, dt$ (min)

σ^2 (Variance) = $\int (t-tm)^2 . t .dt$ $(min)^2$

σ_θ^2 (skewness) = σ^2 / t_m^2 (dimensionless)

$\sigma_\theta^2 = 2/Pe - 2/Pe^2 (1-e^{-Pe})$

By trial & error

Pe = ----------

Graphs:

Plot the graph of C(t) v/s time

Plot the graph of E(t) v/s time

Plot the graph of t E(t) v/s time

Plot the graph of $(t-tm)^2$ E(t) v/s time

8. Inference/discussion

9. Safety precautions

a) The pumps and stirrer is switched off. The needle valve is closed.

b) All liquids in the reactors are drained by opening valves.

c) The power for the control panel is switched off.

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1. **Experiment No.: CC-BTLP901/04**
2. **Experiment Name:** Fermentation in batch, fedbatch and continuous bioreactors.
3. **Objective:**
   i) Enhance participant understanding of fermentation processes and chemistry.
   ii) To find the ability of microorganisms to ferment the given Carbohydrate.
4. **Principle:**

Fermentation is generally defined as the conversion of carbohydrates to acids or alcohols. The conversion of corn sugar (glucose) to ethanol by yeast under anaerobic conditions is the process used to make the renewable transportation fuel, bioethanol. A fermentor is operated by inoculating a complex sugar medium with a microorganism. This microorganism is generally allowed to reproduce under aerobic conditions before the fermentor is switched to anaerobic conditions to produce secondary metabolites such as ethanol.

Carbon sources such as glucose ($C_6H_{12}O_6$) serve two purposes: material building blocks for biosynthesis and for energy. In this experiment, yeast uses the glucose in three ways:

Energy: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + Energy$

Growth (anaerobic or aerobic): $C_6H_{12}O_6(+O_2) \rightarrow Biomass$

Secondary metabolites: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH(Ethanol) + 2CO_2$

The fermentation process occurs in in batch, fedbatch and continuous bioreactors. These experiments will track two parameters throughout the fermentation: glucose concentration and ethanol concentration. Glucose and ethanol concentrations will be analyzed by the titration method and using the spectrophotometer to collect OD (optical density) readings. With these data, the amount of glucose consumed by each pathway can be calculated.

Batch fermentation mode

This mode considered as the simplest lab operation system by using the closed vessel, where all of the medium requirements were added at the beginning of fermentation process to scale-up the microbial biomass production hence its metabolites production. The microbial metabolites may be produced at a primary or secondary stage of the microbial cultivation period.

Fed-batch fermentation mode

This mode will be started with batch fermentation phase until consumption of one or more substrates, nutrients and/or inducers into a bioreactor; the fresh medium was added by using different feeding regimes. Feeding regime can be added via a fixed volume or variable volume of a fresh medium or substrate only during the time course of the process. This feeding strategy can be added continuous or exponentially or pulses over a short or long period during the run. This feeding regime was controlled by feedback control that requires accurate monitoring and operator control to prevent the repressive effects of high substrate concentrations and avoids catabolism repression.

Continuous fermentation mode

This technique has not been widely used in lab scale but common in the industrial scale. The feeding regime prolonging the exponential phase by adding the microbial cells with fresh nutrients and the cells are removed from the bioreactor at the specific rate and time. There are several control techniques will be used through this mode such as the chemostat restricted by the availability of a limiting substrate, the turbidostat operated under no limitations and the last one include the auxostat since the feed rate controlled a state variable (pH, dissolved oxygen). Therefore, it is necessary to calculate the dilution rate, specific growth rate, a yield of the product by using a number of equations.

5. **Materials**
   i) Nutrient broth
   ii) Sterile petriplates
   iii) Micropipettes
   iv) Cuvette
   v) Conical flask
   vi) Sterile tips
   vii) Culture – Overnight culture of *S.cerevisiae.*
   viii) UV Spectrophotometer

6. **Procedure:**

a) Substrate – Biomass enriched with sugar content, Yeast strain *S.cerevisiae.*

b) Sterilization - All glassware are sterilized by autoclaving at pressure 10 lb/inch for a period of 20 minutes.

c) Estimation of Sugar- Total sugar of Biomass is estimated by Anthrone method and spectrophotometric method.

d) Medium for Seed Culture -

Preparation of Basal Media: 1.2g $NaNO_3$, 1.4g $(NH_4)_2SO_4$, 3.0g KH_2PO_4, 0.2g $MgSO_4.H_2O$, 0.05g $CaCl_2$, 0.01g $MnSO_4.7H_2O$, 0.001g $ZnSO_4.7H_2O$, 1.4g Urea, 1% Yeast extract, 2% peptone were added in 500 ml of distilled water and make up the volume to 1000 ml and pH of the media was adjusted to 5.5-6.0. The media was then autoclaved at 121°C and 15 psi for 15 min. 5% dextrose was added after the autoclaving of media.

e) <u>Bioethanol Production</u>

i) 100 ml of this media was then poured in each of 250 ml of flask.

ii) 15g of each sample (biomass) are added and then allowed to incubate for next 72 h in an incubator at 30°C.

iii) Fermented broths will be removed after 72 hours of interval.

iv) Then the broth will be analyzed by distillation and filtration.

f) Distillation and Filtration

i) The mixture of ethanol and hot water were separated by simple distillation unit at a temperature of 78-96°C.

ii) Determination of ethanol content was done by spectrophotometric method.

7. Observations:

This experiment will track three parameters throughout the fermentation: glucose concentration and ethanol concentration. These will be measured using the spectrophotometer to collect OD (optical density) readings.

8. Inference/discussion

9. Safety precautions

a) After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.
b) Keep uninoculated sugar tubes as control tubes.
c) Do not use the tubes with Durham tubes that are partially filled or with bubbles.
d) Over incubation will help the bacteria to degrade proteins and will result give false positive results.

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1. **Experiment No.: CC-BTLP901/05 and CC-BTLP901/06**
2. **Experiment Name:** Kinetics of batch cultivation of yeast
3. **Objective:**
    a) Determination of kinetic parameters for batch cultivation of yeast under shake flask conditions and lab bioreactor.
    b) Determination of growth curve of a supplied microorganism and determines substrate degradation profile and to compute specific growth rate and growth yield from data.
4. **Principle:**

Fermentation is generally defined as the conversion of carbohydrates to acids or alcohols. The conversion of corn sugar (glucose) to ethanol by yeast under anaerobic conditions is the process used to make the renewable transportation fuel, bioethanol. A fermentor is operated by inoculating a complex sugar medium with a microorganism. This microorganism is generally allowed to reproduce under aerobic conditions before the fermentor is switched to anaerobic conditions to produce secondary metabolites such as ethanol.

Carbon sources such as glucose ($C_6H_{12}O_6$) serve two purposes: material building blocks for biosynthesis and for energy. In this experiment, yeast uses the glucose in three ways:

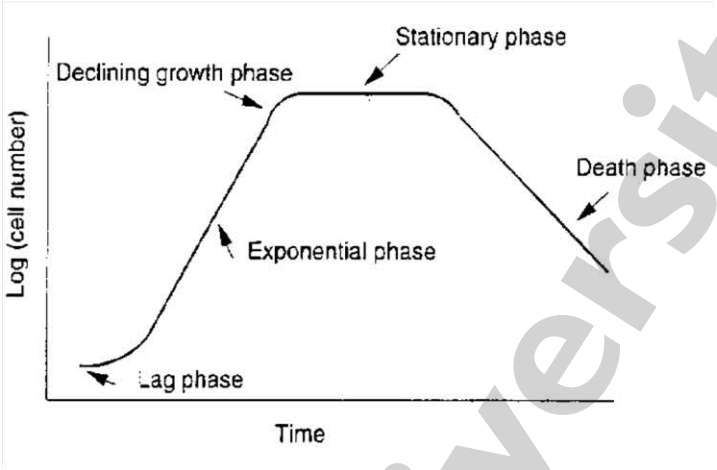
Energy: $\boldsymbol{C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + Energy}$

Growth (anaerobic or aerobic): $\boldsymbol{C_6H_{12}O_6(+O_2) \rightarrow Biomass}$

Secondary metabolites: $\boldsymbol{C_6H_{12}O_6 \rightarrow 2C_2H_5OH(Ethanol) + 2CO_2}$

The fermentation process occurs in under shake flask conditions and lab bioreactor. These experiments will track three parameters throughout the fermentation: glucose concentration, ethanol concentration and Yeast cells. Glucose and ethanol concentrations will be analyzed by the titration method and using the spectrophotometer to collect OD (optical density) readings. Yeast cell mass will be measured using the spectrophotometer to collect OD (optical density) readings. With these data, the amount of glucose consumed by each pathway can be calculated. With these data, the kinetic parameters by each pathway can be calculated.

In batch culture conditions, microbial growth typically follows a five phase cycle from inoculation to cell death. These phases can be seen below as an average over all *live* cells in the population. Growth curves based on OD readings will look slightly different using OD readings since live and dead cells are not distinguishable using absorbance.



The first phase, or *lag phase*, occurs directly after inoculation as the microorganism adapts metabolically to its new environment. The second phase is the *exponential* or *logarithmic growth phase* where cells exhibit *balanced growth*, meaning that cell mass and cell density grow exponentially with time at the same rate.

$$X = X_0 e^{\mu t}$$

X and X_0 are cell mass concentrations (g/L) at time t (hours) and μ (per hour) is the specific cell mass growth rate. The exponential growth period is characterized by a straight line on an *ln*(X) vs. time growth curve. The time to double cell mass, τd (hours), can be calculated from:

$$\tau_d = \frac{ln2}{\mu} = \frac{0.692}{\mu}$$

At some point during the exponential growth phase, the depletion of one or more essential nutrients, or the build-up of toxic growth by-products, reaches a point where rapid balanced growth is no longer possible. During this third phase, the *deceleration* or *declining growth phase*, cells have to again metabolically adapt, this time to increase cell survival potential rather than maximize cell growth. In batch cultures, this phase is generally very short.

The growth of the cells slows until the growth rate is zero (growth rate = death rate) and the cells enter the *stationary phase*. During this time, cells' metabolism shifts from producing primary growth-related metabolites (more cells) to secondary, non-growth, metabolites (ethanol). Cells must break down or *catabolize* their reserves for new energy and building-block monomers to keep up an energized cell membrane, nutrient transport, and cell structure repair. This process is called *endogenous metabolism* and provides *maintenance energy*. In the case of this fermentation, the start of the stationary phase occurs shortly after the reactor is switched to anaerobic conditions, increasing the production of ethanol, which is eventually toxic to the yeast.

The final phase is the *death* or *decline phase*. At this point, the depletion of nutrients or the build-up of toxins is so great that the cell death rate exceeds the growth rate.

5. **Materials**
   a) Nutrient broth
   b) Sterile petriplates
   c) Micropipettes
   d) Cuvette
   e) Conical flask
   f) Sterile tips
   g) Culture – Overnight culture of Yeast strain *S.cerevisiae*
   h) UV Spectrophotometer

6. **Procedure:**

g) Substrate – Biomass enriched with sugar content, Yeast strain *S.cerevisiae.*

h) Sterilization - All glassware are sterilized by autoclaving at pressure 10 lb/inch for a period of 20 minutes.

i) Estimation of Sugar- Total sugar of Biomass is estimated by Anthrone method and spectrophotometric method.

j) Medium for Seed Culture -

Preparation of Basal Media: 1.2g $NaNO_3$, 1.4g $(NH_4)_2SO_4$, 3.0g KH_2PO_4, 0.2g $MgSO_4.H_2O$, 0.05g $CaCl_2$, 0.01g $MnSO_4.7H_2O$, 0.001g $ZnSO_4.7H_2O$, 1.4g Urea, 1% Yeast extract, 2% peptone were added in 500 ml of distilled water and make up the volume to 1000 ml and pH of the media was adjusted to 5.5-6.0. The media was then autoclaved at 121°C and 15 psi for 15 min. 5% dextrose was added after the autoclaving of media.

k) <u>Yeast culture</u>

i) An isolated colony of the organism (*S.cerevisiae.*) was inoculated into 15 ml nutrient broth and kept for overnight incubation.
ii) Following day, the OD of this culture was measured and confirmed.
iii) In order to adjust the OD of the inoculums to the standard value (0.05) the following dilution formula was used

$$OD1V1 = OD2V2$$

Where,

OD1 = OD of the broth culture, inoculated the previous day.
V1 = volume of this broth culture to be added to the inoculums
OD2 = OD of the inoculum (as a standard, this value was adjusted to 0.05)
V2 = volume of the inoculums (in this experiment, 50 ml)

iv) Substitute the values in the equation and V1 was calculated.

v) That much amount (V1) of the inoculums was pipetted out before adding an equivalent amount of the broth to it, so that the net volume remains constant.
vi) The OD was checked at every 30 minutes interval and recorded.
vii) Using this OD value, a standardized growth curve of the organism was plotted. (Absorbance verses time).
viii) Generation time was calculated.

l) Bioethanol Production

i) 100 ml of this media was then poured in each of 250 ml of flask.
ii) 15g of each sample (biomass) are added and then allowed to incubate for next 72 h in an incubator at 30°C.
iii) Fermented broths will be removed after 72 hours of interval.
iv) Then the broth will be analyzed by distillation and filtration.

m) Distillation and Filtration

i) The mixture of ethanol and hot water were separated by simple distillation unit at a temperature of 78-96℃.
ii) Determination of ethanol content was done by spectrophotometric method.

7. Observations:

This experiment will track three parameters throughout the fermentation: glucose concentration, ethanol concentration and Yeast cells. These will be measured using the spectrophotometer to collect OD (optical density) readings.

10. Inference/discussion

11. Safety precautions

i) After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.
j) Keep uninoculated sugar tubes as control tubes.
k) Do not use the tubes with Durham tubes that are partially filled or with bubbles.
l) Over incubation will help the bacteria to degrade proteins and will result give false positive results.

THE NEOTIA UNIVERSITY

1. **Experiment No.: CC-BTLP901/07**
2. **Experiment Name:** Determination of yield factor $Y_{x/s}$ for yeast growth.
3. **Objective:** To determine yield factor $Y_{x/s}$ for yeast growth.
4. **Principle:**

One way to track the fermentation is to calculate yield factors, written as a capital Y with two subscripts denoting product and substrate. For example, $Y_{X/S}$ is the yield factor for amount of cell mass (g of yeast, X) per amount of substrate (g of glucose consumed, S), and is equal to:

$$Y_{X/S} = -\Delta X / \Delta S$$

The yield factor can be calculated at the end of the fermentation to evaluate overall performance, but also for a specific time period during the fermentation to compare the rates of greatest growth. Typical yield factors observed for S. cerevisiae grown aerobically on glucose are around 0.5 g/g, with significantly lower yields under anaerobic conditions.

$Y_{P/S}$ stands for the amount of product (g of ethanol, P) produced per amount of substrate (g of glucose, S) consumed and is calculated the same way:

$$Y_{P/S} = -\Delta P / \Delta S$$

When working with any microorganism, avoiding contamination is vitally important. This prevents other organisms from depleting necessary nutrients and/or producing inhibitory metabolites. Among the aseptic techniques that you will use in this lab are: disinfecting surfaces with 70% ethanol; sterilizing media and equipment under steam pressure in an autoclave; using disposable, radiation-sterilized syringes; and preventing airborne contaminants from entering the fermentor environment by clamping off tubing and attaching 0.2 µm filters, which is smaller than the size of all microorganisms, onto exhaust and inlet vents.

5. **Materials**
   i) Nutrient broth
   ii) Sterile petriplates
   iii) Micropipettes
   iv) Cuvette
   v) Conical flask
   vi) Sterile tips
   vii) Culture – Overnight culture of Yeast strain *S.cerevisiae*
   viii) UV Spectrophotometer
6. **Procedure:**

a) Yeast culture

i) An isolated colony of the organism (*S.cerevisiae.*) was inoculated into 15 ml nutrient broth and kept for overnight incubation.

ii) Following day, the OD of this culture was measured and confirmed.

iii) In order to adjust the OD of the inoculums to the standard value (0.05) the following dilution formula was used

$$OD1V1 = OD2V2$$

Where,

OD1 = OD of the broth culture, inoculated the previous day.

V1 = volume of this broth culture to be added to the inoculums

OD2 = OD of the inoculum (as a standard, this value was adjusted to 0.05)

V2 = volume of the inoculums (in this experiment, 50 ml)

iv) Substitute the values in the equation and V1 was calculated.

v) That much amount (V1) of the inoculums was pipetted out before adding an equivalent amount of the broth to it, so that the net volume remains constant.

vi) The OD was checked at every 30 minutes interval and recorded.

vii) Using this OD value, a standardized growth curve of the organism was plotted. (Absorbance verses time).

viii) Generation time was calculated.

b) Bioethanol Production

i) 100 ml of this media was then poured in each of 250 ml of flask.

ii) 15g of each sample (biomass) are added and then allowed to incubate for next 72 h in an incubator at 30°C.

iii) Fermented broths will be removed after 72 hours of interval.

iv) Then the broth will be analyzed by distillation and filtration.

c) Distillation and Filtration

i) The mixture of ethanol and hot water were separated by simple distillation unit at a temperature of 78-96℃.

ii) Determination of ethanol content was done by spectrophotometric method.

7. Observations:

This experiment will track three parameters throughout the fermentation: glucose concentration, ethanol concentration and Yeast cells. These will be measured using the spectrophotometer to collect OD (optical density) readings.

Then calculate- i)$Y_{X/S}$ is the yield factor for amount of cell mass and ii) $Y_{P/S}$ stands for the amount of product.

12. Inference/discussion

13. Safety precautions

a) After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.
b) Keep uninoculated sugar tubes as control tubes.
c) Do not use the tubes with Durham tubes that are partially filled or with bubbles.
d) Over incubation will help the bacteria to degrade proteins and will result give false positive results.

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1. **Experiment No.: CC-BTLP901/08**
2. **Experiment Name:** Fermentative production of Wine from grapes.
3. **Objective:** To study the effect of sugar content on wine fermentation.
4. **Principle:**

Wine making is still very much an art rather than science. Since many books are devoted to the subject of wine making and wine tasting, the topic will not be elaborated upon here. Although grapes are by far the most often used fruit, various other fruits such as peaches and prunes may also be used to make wines.

The procedures of making grape wine at home are quite straight forward. Grape juice is simply inoculated with a package of yeast starter culture purchased from a supermarket. Primary fermentation lasts for approximately one week; during that time most of the sugar originally present in the juice is converted to ethanol and yeast cells, with the evolution of carbon dioxide. The excess yeast cells are then removed from the juice along with other sediment, and a slower secondary fermentation is allowed to proceed to develop the final flavor. Sugar may be added to the original must to achieve the desired alcohol content or to modify the flavor. The type of wine can be classified according to the color of the wine.

5. **Materials**
   a) Graduated cylinder
   b) Test tubes
   c) Erlenmeyer flasks or bottles
   d) Rubber stoppers
   e) Tygon tubing
   f) Hydrometer
   g) Balance
   h) Grape juice
   i) Sucrose
   j) Active dry wine yeast, strains of *S.cerevisiae.*
6. **Procedure**

a) Prepare Starter Yeast Culture

i) Mix 1 g of dry wine yeast culture in 100 ml of grape juice.
ii) Let the yeast grow in a loosely capped container at room temperature for 24 hours.

b) Primary Fermentation

i) Add enough sugar to grape juice to prepare the following 4 substrates, about 1 liter each:

Run	Conc. of Extra Added Sugar
A	0.0g/l
B	100.0g/l
C	200.0g/l
D	300.0g/l

ii) Measure the specific gravity and PA value for each of the starting substrates with a hydrometer. This is the initial PA value which will be used later to estimate the alcohol content.
iii) Inoculate each bottle with 20 ml of the starter yeast culture prepared in the previous step.
iv) Plug the juice bottle with a rubber stopper. A piece of Tygon tubing is extended from the stopper to provide a vent for the evolved carbon dioxide. The other end of the tubing is dipped in water in a small test tube taped to the bottle. The water prevents the entry of oxygen, which alters the metabolism of the yeast and spoils the wine. At the same time, carbon dioxide can escape from the bottle.
v) Ferment at room temperature for one week.

c) Secondary Fermentation

i) At the end of one week, decant the juice from the bottle to clean individual temporary containers.
ii) Measure the PA values for each of the substrate with a hydrometer. Estimate the alcohol content by subtracting the present PA value from the initial PA value.
iii) Discard the sediment and wash each bottle with water.
iv) Pour the juice back into the cleaned bottle. Put back the cleaned assembly of rubber stopper and Tygon tubing.
v) Ferment slowly for another 4-6 weeks.
vi) Measure the PA values as before when it is ready for consumption.

7. Observations

a) Measure the alcohol % by spectrophotometer.
b) Taste the wine.
c) Try out other fruit juices.

8. Inference/discussion

9. Safety precautions

a) After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.
b) Keep uninoculated sugar tubes as control tubes.
c) Over incubation will help the bacteria to degrade proteins and will result give false positive results.
d) Selection of suitable starter cultures for malolactic fermentations and the reduction of arginine concentrations in the grape.
e) Urease was introduced in 1997 by the EU as a new enzymatic wine treatment agent and can be used in exceptional cases.
f) The enzyme splits urea into ammonia and carbon dioxide, preventing urethane formation.

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1. **Experiment No.: CC-BTLP901/09**
2. **Experiment Name:** Isolation of microorganisms from soil samples.
3. **Objective:** To isolate and count the microorganisms found in a sample of soil by the dilution method using aseptic techniques.
4. **Principle:**

   Soil contains varieties of microorganism including bacteria that can be established in any natural environment. Bacteria are the most important and abundant microorganism which is present in surrounding environment. These are very small, unicellular, primitive and non chlorophyll containing microorganism. Dilution method is one of most important method to isolate the soil bacterium which allows the list of living cells in the soil. An enzymatic activity of one bacterium differs from another bacterium. Biochemical test is used to differentiate among the other bacteria. 16S rRNA gene sequencing studies the bacterial taxonomy and phylogeny.

   Alcaligenes sp are motile gram negative soil bacterium which can be able to produce exo poly saccharide such as curdlan and welan gum. In this work we focused to isolate the exo polysaccharide producing soil bacterium.
5. **Materials:**
   i. Erlenmeyer flask containing 50ml of sterile agar (0.1%).
   ii. 1 cup containing 0.5g of soil
   iii. 4 small vials containing 4.5ml of sterile agar (0.1%)
   iv. 5 sterile 1ml pipettes.
   v. 1 sterile glass stirring rod
   vi. 6 Petri plates containing about 10ml of PDA (potato dextrose agar)
   vii. 6 strips of Parafilm
   viii. paper towel
   ix. disinfectant
   x. marking pens
6. **Procedures:**
   a) **Soil sample collection**: For bacterial isolation, 10 g of soil was collected from different area near campus from upper layer of the farmland where maximum population of microorganism was concentrated. 5 g of soil sample was collected by using clean and dry sterile spatula in a clean polythene
   b) Mark the dilutions on the vials (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and Petri plates (10^{-4}, 10^{-5}, 10^{-6}).
   c) Add the 0.5g soil to the Erlenmeyer flask containing 50ml of agar (this is the 10^{-2} dilution).
   d) Shake well for at least one minute.
   e) Using a sterile pipette, take 0.5ml and place it in a vial containing 4.5ml of 0.1% agar. (this is the 10^{-3} dilution).
   f) Shake well for at least one minute.
   g) Repeat steps 4 and 5 for the three other dilutions (10^{-4}, 10^{-5}, 10^{-6}).

h) Starting with the weakest dilution (10^{-6}), pipette 1ml onto each of 2 Petri plates containing PDA. Spread over the entire surface using the sterile glass stirring rod. (The 10^{-6} dilution first.) Follow the same steps for the 10^{-5} and 10^{-4} dilutions.

i) Seal the Petri plates with the Parafilm.

j) Incubate the Petri plates at room temperature.

k) Observe after 24, 48 et 72 hours.

7. Observations:

a) Record the number of colonies/Petri plate/dilution.

b) **Biochemical identification**

Characteristics	Observation	Interference

c) Calculate the number of microorganisms per gram of soil using the following formula:

# of colonies/plate × the dilution factor = # organisms / 1 gram of soil

d) Record the characteristics of each colony.

8. Inference/discussion

9. Safety precautions

a) Prevent contamination and possible infection from the microorganisms.

b) Make sure to properly dispose of all Petri plates, Soil solutions and pipettes.

c) This procedure only measures cultivatable bacteria.

THE NEOTIA UNIVERSITY

1. **Experiment No.: CC-BTLP901/10**
2. **Experiment Name:** Production of Industrially important Enzyme by submerged fermentation (Lab scale).
3. **Objective:**
   i) To screen the amylase producing bacteria from the soil.
   ii) To produce extracellular amylase by submerged fermentation.
   iii) To know the assay methods of amylase enzymes.
4. **Principle:**

Screening α-amylase producers

The isolation of microorganism is potentially important before being screened for their production of enzymes of interest. Amylase producing microbes are ubiquitous and are usually obtained from soil sediments. Two methods are generally useful for screening of α-amylase producers; they are 1) solid-media based, or 2) aqueous mediabased techniques. The common method is standard plating technique on the solid media with starch as the substrate. The second method (substrate selection) is through enrichment before screening of the isolate on solid media, where efficient strains are isolated according to their affinity for a particular substrate. Through these methods, different bacteria and fungi have been screened and studied for amylase production.

In this experiment, serial dilution based method can be used to screen the amylase producing bacteria. Subsequently the enzyme production is quantified based on the amount of resulting end product produced by the dinitro salicylic acid (DNS) and Nelson-Somogyi (NS) techniques. In the solid-agar method, the appropriate strain (bacteria) is inoculated onto the starch-containing agar at the center of the Petri plate. After an appropriate incubation period, the plate is flooded with iodine solution, which reveals a dark bluish color on the substrate region (starch-iodine complex) and a clear region (due to hydrolysis) around the inoculum, indicating the utilization of starch by the microbial amylase. Further, pure culture of large hydrolytic zone producing bacteria will be grow in aqueous media with starch (1 to 2% w/v), subsequently total enzyme productivity will be quantified by DNS based method. In this method enzyme and the starch substrate are mixed and incubated for 10 min at 50°C. Then, cool to room temperature, the absorbance of the solution is read at 540 nm. This method is to detect the release of reducing sugars from substrate hydrolysis by amylase enzyme.

Submerged Fermentation

In this method mineral media or the nutrient media supplemented with high starch substrate is used. Bacteria growing in submerged fermentation produce extracellular amylases and depolymerize the starch in to simple sugars for its growth and energy requirements. The amount of enzyme production will be quantified and the productive capacity of the organism can be determined. In this experiment, culture supernatant can be used as enzyme source, using this reaction will be performed with standard starch

solution at a standard time period. During this reaction enzyme react on the starch substrate to produce maltose sugars, described below.

Amylase activity can be estimated by the analysis of reducing sugar (maltose) released during hydrolysis of 1% (w/v) starch. The amount of maltose is estimated by 3,5-dinitrosalicylic acid method. One unit of amylase activity is defined as the amount of enzyme that release 1mMol of reducing sugar as glucose per min under assay conditions. Enzyme activity is expressed as the specific activity, which represents as U/ml.

DNS method Principle

Reducing sugars have the property to reduce many of the reagents. One such reagent is 3,5-dinitrosalicylic acid (DNS). 3,5-DNS in alkaline solution is reduced to 3 amino 5 nitro salicylic acid.

5. Materials:

i. Media: Starch agar, starch broth, nutrient agar, nutrient broth.
ii. Reagents: Iodine solution, DNS reagent
iii. Sample: Soli sediment.
iv. Microorganism: Pure culture of *Bacillus amyloliquifaciens* (or) *B. licheniformis* (or) *Bacillus subtilis.*
v. Buffer: 0.02 M sodium phosphate buffer (pH, 6.9) with 0.006 M sodium chloride.
vi. Starch solution: 1.0% starch solution in 0.02 M sodium phosphate buffer (pH, 6.9).
vii. Maltose stock solution: 1mg/ml, stored at 40C.
viii. Others: Laminar hood, burner, pipettes, Erlenmeyer flask, Shaking incubator, test tubes, sterile water blanks, Spectrophotometer, cuvettes, etc.

6. Procedure:

a) Screening α-amylase producers

i. Prepare starch agar plates.

- Prepare ten starch agar plates (sterile) with 1% starch as carbon and energy source.
- Mark the media plates and keep this in the inoculation chamber.

ii. Sample preparation and serial dilution.

- Take one gram of soil in to a 10 ml of sterile water blank. Vortex the content and allow to settle down for the soil particles.

- Perform a serial dilution procedure to transfer one ml of the soil suspension to the second tube and mix thoroughly.
- Repeat the step up to 10^{-7} dilutions.

iii. Plating the sample

- Take 0.1 ml of the diluted soil samples (10^{-4} to 10^{-7}) and inoculate on the sterile starch media.
- Using L-rod to spread the sample at the entire surface of the media.
- Incubate the plates at 32 to 37oC for 48 hours.
- Observe the growth of the bacteria after the incubation period.
- After the bacterial growth the agar surface is flooded with iodine solution for 30sec. decant the excess iodine and observe the clear transparent zone around the bacterial colony, with dark blue background.

b) Submerged fermentation of α-Amylase

i. Bacterial inoculum is prepare in nutrient broth inoculated with a loop full of bacteria from the mother slant and incubate it for 24 hours to reach log phase growth (10^8 to 10^9 cfu/ml).
ii. Prepare 100 ml of media in 250 ml Erlenmeyer flask for amylase fermentation and sterilize it [a nutrient medium supplement with 1% (w/v) starch as carbon and energy source].
iii. Aseptically transfer 1.0 ml of log phase inoculums to the fermentation medium.
iv. Incubate the flask for 24 to 48 hours at incubated shaker (35oC).
v. After incubation, centrifuge the fermented media at 8000 rpm for 10 min.
vi. Transfer the supernatant to the fresh tube for enzyme assay. Count the total number of colonies and the starch hydrolytic colonies.
vii. Measure the zone of starch clearance with standard ruler.
viii. Select any five

c) α-Amylase Enzyme assay

Preparation of the D.N.S. reagent: 10 g of 3,5-dinitrosalicylic acid are gradually dissolved under heating conditions, in 700 ml of NaOH solution 0.5 N. Then 300 g of sodium potassium tartrate (NaKC4O6.4H2O) are added and distillated water was added in the mixture until a final volume of 1000 ml. The reagent has a dark orange colour and is stable for several days in room temperature.

d) Prepare standard graph for maltose as follows:

i. Standard solutions of maltose (0-10 μmoles/l) are prepared in test tubes.
ii. 1 ml of D.N.S. reagent is added in each tube and the mixture is
iii. Agitated for a few seconds on vortex mixer.
iv. The samples are placed in a water bath (T=100°C) for 5 min and then they are left to cool at room temperature.
v. 5 ml of deionized water are added in each sample, followed by agitation.
vi. The absorbance (A) of the samples is measured at λ=540 nm.
vii. A standard curve is being drawn.

e) Enzyme reaction is performed in a test tube described below;

i. Pipette out 0.5 mL enzyme solution (step-1 6th point) and incubate tubes at 25 oC for 3 min.
ii. Add 0.5 mL starch solution and incubate for 5 min (Room Temperature).
iii. Stop the reaction by adding 1 mL DNS reagent.
iv. Heat the solution in a boiling water bath for 5 min.
v. Cool it in running tap water.
vi. Make up the volume to 10.0 mL by the addition of distilled H_2O.
vii. Blank is prepare without enzyme, and follow the above steps.
viii. Read the absorbance at 540 nm using UV-Vis Spectrophotometer.

7. Observations:

Qualitative determination of α-amylase producer

Sl. No	Dilution factor	Plate name	Number of colonies [CFU]		% of starch hydrolytic colonies
			Total CFU	Starch hydrolysis CFU	

Calculation: % Starch hydolytic organisms = Number of CFU with clear zone x 100 / Total number of colonies

Note:

Positive result: *Following bacterial inoculation and incubation, bacterial colony developed with colourless clear hydrolytic zone.*

Negative result: *The bacterial colony without clearing zone*

Sugar Standard Graph

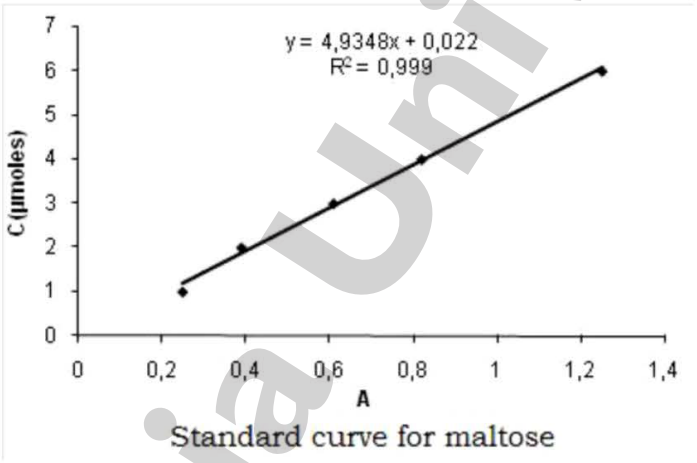
Sl. No	Concentration of Glucose (mg/ml)	OD at 540 nm

Calculation of α-amylase activity

One unit (U/mL) of α-amylase activity is defined as: the amount of protein (α-amylase) required to liberate 1 µmol (0.18 mg equivalence) of reducing sugar (D-glucose) from starch/min, under the assay conditions.

$$\text{Formula: } \alpha\text{-amylase activity (U/mL)} = \frac{\Delta E \times Vf}{\Delta t \times \sum \times Vs \times d}$$

ΔE = Absorbance at 540 nm
V_f = Final volume including DNS
V_s = Volume (mL) of α-amylase used
Δt = Time of hydrolysis
Σ = extinction coefficient = 0.165 cm^2/µm glucose
d = diameter of the cuvette = 1



Standard curve for maltose

8. Inference/discussion

The amount of enzyme produced by the bacteria at the give time period is = _____ U/ml.

9. Safety precautions

d) Prevent contamination and possible infection from the microorganisms.
e) Make sure to properly dispose of all Petri plates, Soil solutions and pipettes.
f) This procedure only measures cultivatable bacteria.

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