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Research Paper

Kinetic studies on Production of Biosurfactant (Trehalose lipid) by Nocardia Hydrocarbonoxydans

Engineering

KEYWORDS :

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ABSTRACT

Surfactants of biological origin are gaining importance in recent years due to their biodegradability and nontoxic nature. In present work the synthesis of trehalose-lipid biosurfactant by Nocardia hydrocarbonoxidans NCIM-2386 was studied in batch experiment. Since the concentration of carbon source plays a major role in the synthesis of biosurfactants, the experiment was conducted in different initial sucrose concentrations ranging from 10g/l to 60g/l in intervals of 10g/l keeping all other medium ingredients same. The optimum concentration of substrate (surcrose) for the production of biosurfactants (Tehalose-lipid) was found to be 50g/l.In this work an attempt has been made to understand the kinetics of biomass growth & product formation. The Logistic model for biomass growth has predicted well for higher initial substrate concentrations of 50g/l and 60g/l. In the present batch cultivation studies the biomass yield was found to be (Yx/s) = 0.13 g dry weight of cells/g of substrate consumed. The product formation kinetics was predicted favorably with Leudeking-Piret model for higher concentrations of sucrose ie 50g/l and 60g/l. It was evident from α and β values that the product formation kinetics is more oriented towards growth-associated type.

Introduction

Surfactants are surface active agents capable of reducing surface and interfacial tension at the interface between liquids, solids and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids. The enormous market demand for surfactants is currently met by numerous synthetic, mainly petroleum based, chemical surfactants. These compounds are usually toxic to the environment and non-biodegradable.

Biosurfactants are structurally diverse group of surface active molecules synthesized by microorganisms. They posses clearly defined hydrophilic and hydrophobic groups & hence they are called as amphiphilic compounds. They have advantage over their chemical counterpart because of non toxic nature, biodegradability and effective at extreme temperature and pH conditions.

Biosurfactants are mostly combinations of lipids, sugars and proteins. Microbial surfactants are known for their usefulness in enhanced oil recovery (Parkinson et al., 1985. Finnerty et al., 1983). Further they are almost as effective in application as many conventional synthetic surfactants. Bacterial surfactants are known to reduce surface tension of aqueous solutions to about 27 mN/m and interfacial tension against octane or decane, to 10-12 mN/m, thereby competing favourably with synthetic surfactants (Lin et al., 1996). The usage of biosurfactant through bulk production however depends chiefly on the economical feasibility of such operations.

Biosurfactant Assay for Optimization studies:

The effective biosurfactant assay for optimization studies should have the capability of handling large amount of samples with relatively good specificity and sensitivity. So far the most widely used methods for the detection of biosurfactants have been interfacial/surface tension measurements are good for preliminary studies, because interfacial/surface tension of a cell-free culture against organic phase are generally strength affected by factors such as pH and ionic strength, which excludes their utility as a quantitative assay to investigate the effect of these factors on biosurfactant production.

Biosurfactants possess some advantages over synthetic surfactants. By virtue of properties of biodegradability, substrate specificity, lower toxicity ,chemical and functional diversity, and rapid/ controlled inactivation, biosurfactants are gaining importance in various industries like agriculture, cosmetics, food, textiles, petrochemicals, etc

One of the earliest applications of biosurfactants to be recognized & industrial attention drawn was in enhanced oil recovery (EOR), oil spill applications & waste water treatment. Following conventional recovery methods, about 50% of crude remains in reservoir. Surfactant-based technology is being developed to enhance the recovery of remaining 50%. The current research is aimed at optimization of either in-situ application of biosurfactants (Finnerty et al., 1983). The main importance of biosurfactants is that they facilitate the growth of microorganisms on hydrocarbons & a large number of useful products can be obtained from these substrates (Abbott et al., 1971).

Materials and methods:

Microorganism: *Nocardia hydrocarbonoxydans* (NCIM-2386) is an actinomycetes, chosen for the present study by virtue of its effectiveness to degrade hydrocarbons. It is procured from National Collection of Industrial Microorganisms (NCIM), a division of National Chemical Laboratories, Pune. The strains are periodically sub cultured once in 15 days on agar slants and are stored at 4^0C.

Kingdom:	Bacteria
Phylum:	Actinobacteria
Order:	Actinomycetales
Suborder:	Corynebacterineae
Family:	Nocardiaceae
Genus:	Nocardia

Nocardia is a genus of Gram-positive, catalase-positive, rod-shaped bacteria; some species are pathogenic (nocardiosis). *Nocardia* are found worldwide in soil that is rich with organic matter.

Inoculum preparation: About 5 % inoculum from cultures grown on medium as mentioned in table 1.1. This culture at about 48 hours was found to be in the exponential growth phase and hence was used as inoculum to batch experiments with different sucrose concentrations.

Table 1.1 Medium components and their concentrations:

Medium components	Concentration
Sucrose	10 to 60 g/l
$NaNO_3$	1 g/l
$MgSO_4 . 7H_2O$	0.1 g/l
Yeast extract	0.2 g/l
K_2HPO_4	0.1 g/l
$CaCl_2$	0.1 g/l

Cultivation: Shake flask cultivation was performed with 100ml of culture volume taken in 250ml Erlenmeyer flasks. Flasks were placed on rotary shaker at a speed of 120 rpm without temperature control. Fermentation was allowed to proceed up to 192 hours. The initial pH in all cases was adjusted to 6. The pH was uncontrolled during all fermentations.

Sampling: Sample of suitable volume were removed every 24 hour and preserved at 4°C for analysis. Samples were centrifuged at 10000 rpm for 15 min. The supernatant was analyzed for trehalose lipid and sucrose. The residue was analyzed for cell biomass after washing and drying to constant weight.

Determination of biomass: Dry Centrifuge tubes were taken and their empty weight was determined.12 ml of culture broth was centrifuged at 10000 rpm for 10 min. The supernatant was removed completely and collected in separate test tube. The Centrifuge tube containing wet biomass (cell pellets) is kept for drying in a hot air oven at an interval of one hour. After every hour the Centrifuge tube is weighed and the value is reduced by its empty weight. The drying is continued till we get a constant value of dry biomass.

Estimation of Sucrose by Anthrone reagent method:
0.1ml of supernatant is taken in a test tube.It is diluted to 10 ml using distilled water.0.1 ml of this diluted solution is taken and diluted to 1 ml using distilled water.To this 4 ml of 2g/l concentration of anthrone reagent is added, mixed well and Kept in a boiling water bath for 8 min. The absorbance is taken at 660nm using a spectrophotometer (Hedge J E et al., 1962).

Estimation of Trehalose lipid:
The trehalose-lipid was estimated by Phenol-Sulphuric acid method (Dubois et al., 1956). This method is sensitive to micro quantities of sugar present in the sample. Biosurfactant concentrations were measured as trehalose-lipid.

To measure the concentration of trehalose-lipid in the fermentation broth, the supernatant obtained after centrifugation was adjusted to pH 2. It was subjected to liquid-liquid extraction using chloroform and methanol in the ratio of 2:1. The extract was kept at room temperature till the solvent was evaporated completely.

The residue was dissolved in 0.1N $NaHCO_3$ and the trehalose-lipid concentration was measured as below:

1 ml of suitably diluted sample prepared as mentioned above was treated with 1 ml of 5% phenol and shaken well.5 ml of concentrated sulphuric acid was added rapidly. The stream of acid being directed against the liquid surface rather than against the sides of test tube in order to obtain good mixing and the tubes were kept at the room temperature was 10 min. Then the tubes were kept in the water at room temperature for color to develop, after which absorbance was read at 480nm in a spectrophotometer.A graph of absorbance Vs standard concentration of trehalose was plotted in the concentration range of 0 to 100 mg/ml.Trehalose-lipid were estimated by Phenol-Sulphuric acid method and expressed in terms of trehalose.

Results and discussion
As in case of several bioproducts the synthesis of biosurfactants is also known to be influenced by the concentration of carbon source. The types of sugar influences the hydrophilic moiety of glycolipid. Alkanes are also known to decide the type of fatty acid moieties present in the gycolipid (Lin et al., 1996.). The effect of sucrose on product formation was studied for the six different initial substrate concentrations ranging form 10g/l to 60g/l at an intervals of10g/l keeping all other medium ingredients same. An attempt has been made to understand the kinetics of biomass growth and product formation.

The logistic model, which is known to predict the biomass growth for entire growth cycle is used in the present work.

The Leudeking-Piret model is used to predict product formation kinetics. This model is used to predict whether the product formation is growth associated or non-growth associated.

The results presented here and the discussion aims to describe the production of biosurfactant during the course of fermentation as a function of time and the kinetics of biomass growth and product formation.

Batch data for biomass, trehalo-lipid and sucrose concentrations using different concentration of carbon source:
During batch fermentation experiments using *Nocardia hydrocarbonoxydans* (NCIM 2386) significant amount amounts of the product trehalose-lipid was detected in the exponential phase and in stationary phase. Initial studies were aimed at obtaining the effect of initial substrate concentration on the biomass and product concentrations.

Fig 1.1 (a) (b) (c) shows the pattern of biomass growth, sugar utilization and trehalose-lipid production profiles for initial sucrose concentration of 10g/l. The biomass reached stationary phase at about 85-90h of fermentation. The maximum biomass concentration was 5.6g/l. The stationary phase lasted for about 85-120hr, after which biomass entered the Declining phase. The final biomass concentration at the end of the fermentation at 192 h was 4.5 g/l. Sugar utilization rate was very rapid till 24 h of fermentation. About 50% of sucrose was consumed at the end of 50 h. The residual sucrose concentration at the end of 192 h was 0.9 g/l. The maximum concentration of trehalose-lipid was 16mg/l, produced during the stationary phase of fermentation.

The profiles of biomass growth, sugar utilization and trehalose-lipid production for the run with 20 g/l glucose are shown in the fig. 1.2 (a) (b) (c) The maximum biomass concentration was 5.8 g/l. Sugar utilization rate was very rapid till 48 h of fermentation. Nearly 50% sucrose was consumed at the end of 75 h. At the end of fermentation 2.5 g/l of sucrose left in the medium. The rate of trehalose-lipid production increased slowly and reached the value 26mg/l at about 192h.

Fig 1.3 (a) (b) (c) below gives the results of initial sucrose concentration 30 g/l. The pattern of biomass growth was similar to that of 20 g/l but the maximum biomass concentration was increased to 7.5 g/l. About 50% of sucrose was consumed at the end of 72h of fermentation. Sucrose left in the medium at the end of fermentation was about 9.5 g/l trehalose-lipid production increased slowly and became steady after 108 h of fermentation. The maximum production of about 26mg/l was observed at about 192 h.

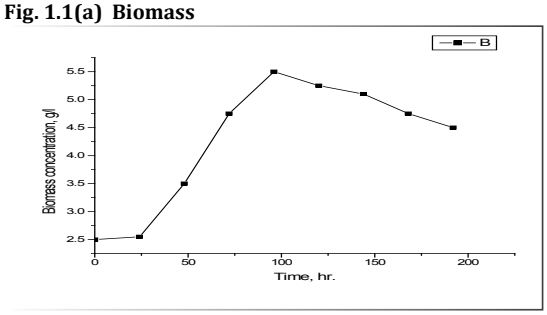
The profiles of biomass growth, sugar utilization and trehalose-lipid production for the run with 40 g/l sucrose are shown in the fig. 4.4 (a) (b) (c). The maximum biomass concentration was 7.16 g/l. Sugar utilization rate was very low compared to

other cases. Nearly 50% of sucrose was consumed at the end of 48 h. At the end of fermentation 8.5 g/l of sucrose was left in the medium. The rate of trehalose-lipid production increased slowly and reached the value of 22.5 mg/l at about 192 h.

Fig 1.5 (a) (b) (c) gives the results of initial sucrose concentration 50 g/l. The pattern of biomass growth was similar to that of 40 g/l but the maximum biomass concentration was increased to 6.4 g/l. About 50% of sucrose was consumed at the end of 50 h of fermentation. sucrose left in the medium at the end of fermentation was about 11 g/l. Trehalose-lipid production increased slowly and became steady after 108 h of fermentation. The maximum production of 29mg/l was observed at about 192 h.

Fig 1.6 (a) (b) (c) gives the results of initial sucrose concentration 60 g/l. The pattern of biomass growth was similar to that of 50g/l but the maximum biomass concentration increased to 6.6g/l. about 50% of sucrose was consumed at the end of 50 h of fermentation. sucrose left in the medium at the end of fermentation was about 10.5 g/l. Trehalose-lipid production increased slowly and became study after 108 h of fermentation. The maximum production of about 28mg/l was observed at about 192 h.

Fig. 1.1(a) Biomass



Batch cultivation with initial sucrose concentration of 10g/l ; Initial pH:6.0 at ambient temperature.

Fig. 1.1(b) Sucrose

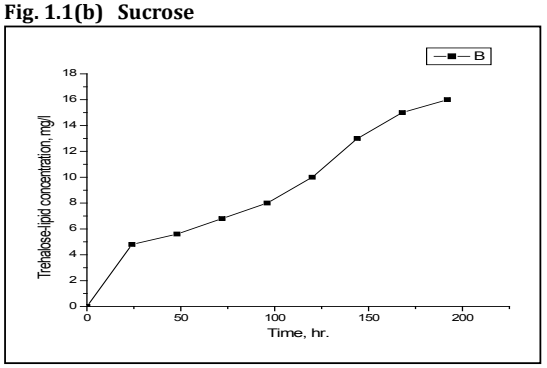
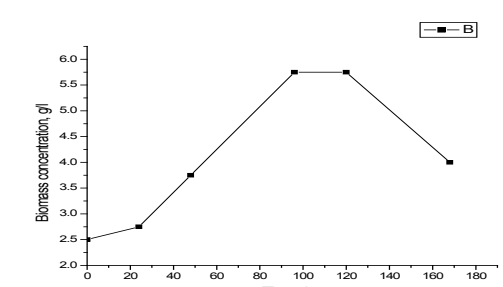


Fig. 1.1(c) Trehalose-lipid



Batch cultivation with initial sucrose concentration of 20g/l ; Initial pH:6.0 at ambient temperature.

Fig. 1.2 (a) Biomass

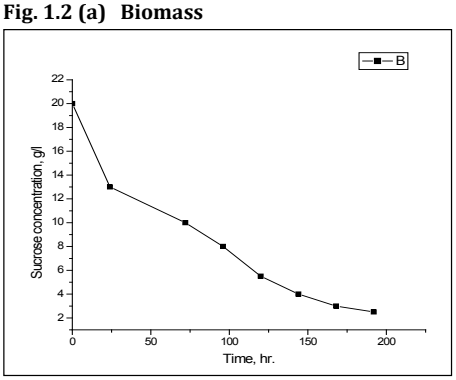


Fig. 1.2 (b) Sucrose

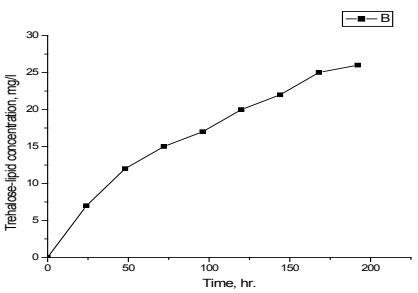


Fig. 1.2 (c) Trehalose-lipid
Batch cultivation with initial sucrose concentration of 30g/l ; Initial pH: 6.0 at ambient temperature.

Fig. 1.3 (a) Biomass

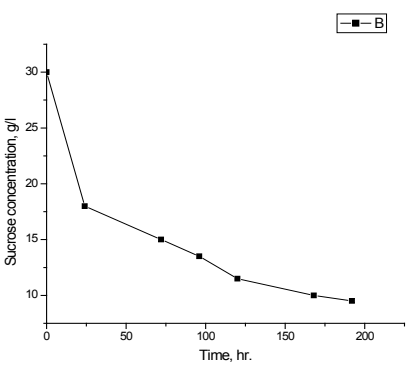


Fig. 1.3 (b) Sucrose

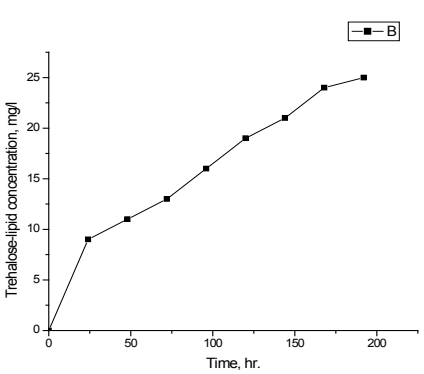


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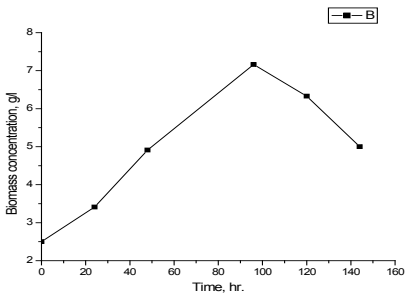
Fig. 1.3 (c) Trehalose-lipid



Batch cultivation with initial sucrose concentration of 40g/l ; Initial pH: 6.0 at ambient temperature.

Fig. 1.4 (a) Biomass

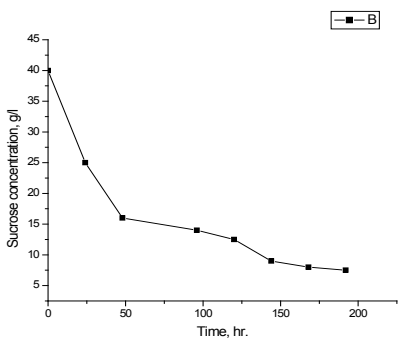


Fig. 1.4 (b) Sucrose

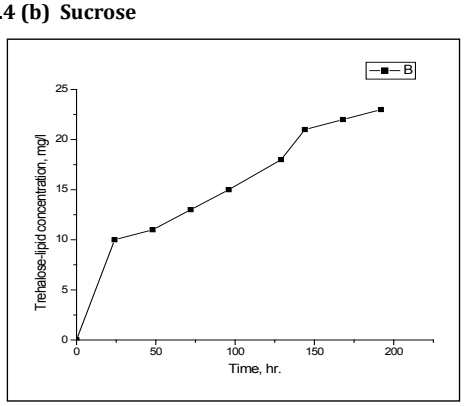
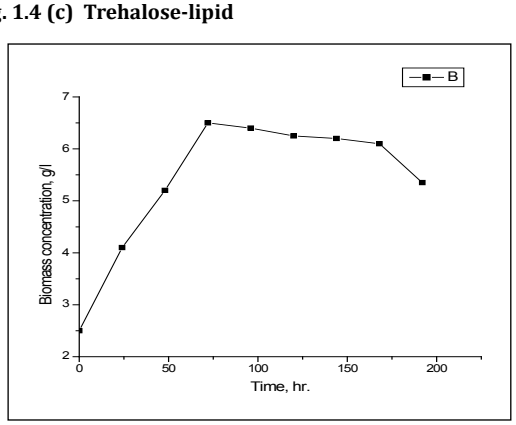


Fig. 1.4 (c) Trehalose-lipid



Batch cultivation with initial sucrose concentration of 50g/l ; Initial pH: 6.0 at ambient temperature.

Fig. 1.5 (a) Biomass

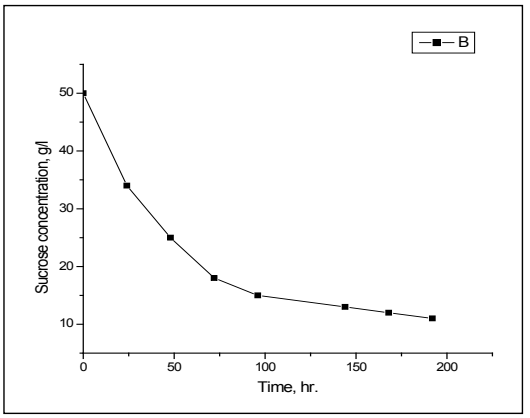


Fig. 1.5 (b) Sucrose

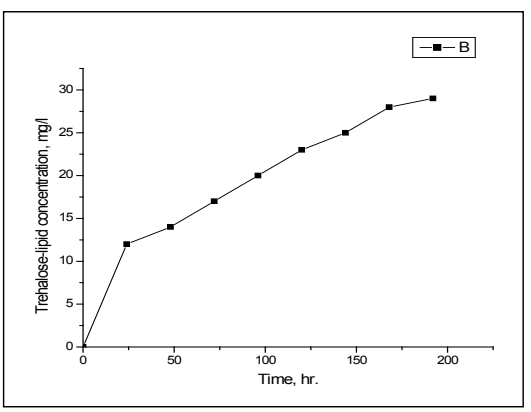
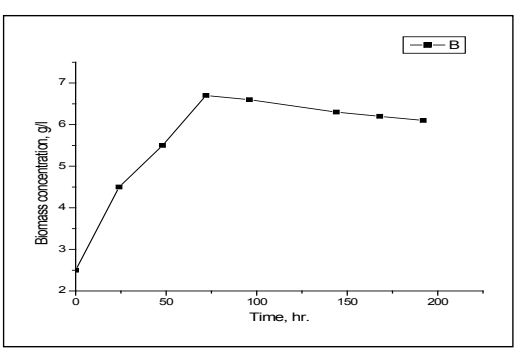


Fig. 1.5 (c) Trehalose-lipid



Batch cultivation with initial sucrose concentration of 60g/l ; Initial pH: 6.0 at ambient temperature.

Fig. 1.6 (a) Biomass

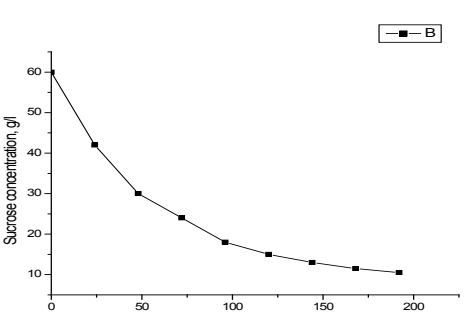


Fig.1.6 (b) Sucrose

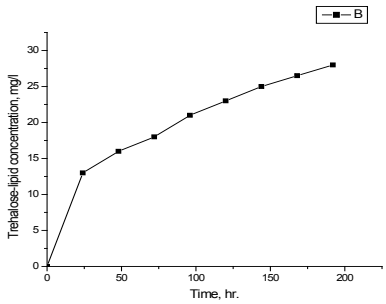


Fig.1.6 (c) Trehalose-lipid

Growth Model
Models of Batch Growth Kinetics:
Monod's Model:
Simple growth kinetics can be modeled using the Monod's expression which relates the growth rate to the initial substrate concentration.

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

This expression is suitable for cases where the specific growth rate approaches its asymptote at high initial substrate concentrations, such as, for growth patterns which are free from substrate induced inhibition. However, for simple growth kinetics using the Monad's model, μ approaches μmax, for too slowly with initial substrate concentration to be a proper representation of experimental data. Monod's model although useful due to its simplicity, is inadequate over the whole batch growth cycle i.e. the lag phase, post-exponential phase and the death phase. Other expressions for μ are more useful, such as the logistic equation.

The Logistic Model:
The logistic equation, which originates from the exponential growth law is given by,

$rx = dX/dt = \alpha\ X\ (1- X / \beta)$

Where α and β are dimensional constants, holds for the entire cycle. The rate of change in cell mass concentration in the death phase is proportional to X2 (La motta et al., 1976) gave forms for α and β as μmax and Xmax respectively, so that

$dX/dt = \mu max\ X\ (1- X/ Xmax)$
$dX/X(Xmax – X) = (\mu max / Xmax)\ dt$
On integrating by partial fractions, we get,
$\ln [X / (Xmax – X)] = \mu max\ \ t\ +\ \ln [Xo / (Xmax – Xo)]$

According to above equation, a plot of ln (X / Xmax – X) Vs t should give a straight line with slope μmax if it fits the experimental data. Xmax is obtained from experimental data. The value of Xo is determined from the intercept. Plots of ln [X / (Xmax – X)] Vs t for different initial sucrose concentrations, shown in Figs. 1.7 (c) (d) (e) (f) indicate a satisfactory linear relationship for higher sucrose concentrations. The results were unsatisfactory for lower concentrations of sucrose. Thus indicate the suitability of the logistic model for biomass growth, for higher concentrations of sucrose. The values of μmax and Xo are thus obtained from Figs 1.7 (a) to (f) for all different initial sucrose concentrations. The **μ**max values obtained for all initial sucrose concentration were found to be nearly uniform. A mean value of 0.0153 h^{-1} was taken as μmax.

Yield Coefficient for Biomass Concentration:
The substrate utilization for biomass production is given by, d.
$-dS/dt = (1/Yx/s)\ (dX/dt)$

On integrating the above equation between the boundary conditions, S = So to S, and X=Xo to X, we get,

$-(S-So) = (1/Yx/s)\ (X-Xo)$
On rearrangement,
$X = (So\ Yx/s + Xo)-(S\ Yx/s)$

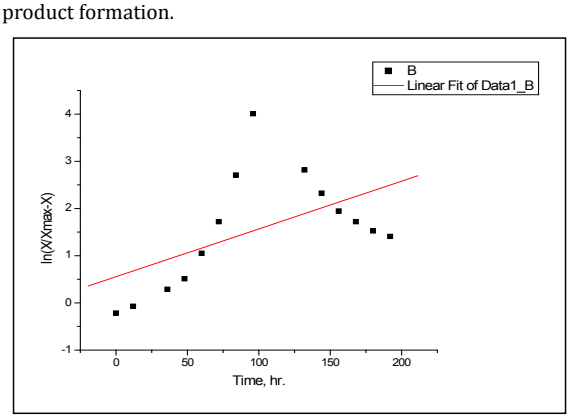
A plot of X Vs S will give a straight line with slope (-Yx/s) and intercept So. Yx/s + Xo). Figs. 1.8 (a) to (f) shows the plots of X Vs S for different initial sucrose concentration from 10g/l to 60g/l. The plots indicate reasonably good linear fits for all the initial concentrations of sucrose. It was found that, regression coefficient greater than 0.8 for 10g/l, 50g/l and 60g/l. Yx/s is obtained from these plots. Fairly constant values for biomass yield coefficient were observed and average value was found to be 0.13 g dry wt. of cells/ g of substrate.

Product Formation Kinetics
Synthesis of biosurfactant took place throughout the exponential growth phase and product peaked well even after the stationary phase. Hence from this we cannot conclude whether the product formation kinetics is growth associated or non-growth associated. Therefore to find out the type of product formation kinetic, Leudeking-Piret model has been used.

Leudeking-Piret Model for Mixed Growth:
The Leudeking-Piret expression relates the rate of product formation to two contributions, a non growth associated term and growth associated term. The later is proportional to the specific growth rate μ, while the former depends on the biomass concentration prevailing at that time (Bailey & Ollis, 1986). This is given as,

$dP/dt = \alpha\ (dX/dt) + \beta\ X$
where, **α** = stoichiometric constant associated with growth associated product formation.
β = proportionality constant associated with non growth type (per hour).
Dividing throughout by X,
$1/X\ (dP/dt) = \alpha/X\ (dX/dt) + \beta$
Hence, $1/X\ (dP/dt) = \alpha\ \mu + \beta$

A plot of 1/X (dP/dt) Vs **μ**, gives a straight line with a slope α and intercept β. 1/X (dP/dt) was plotted against μ at different fermentation times for each run (pertaining to a particular initial concentration of sucrose) to examine the nature of growth associated / non growth associated terms. Figures 1.9 (a) to (f) show these plots. The data had shown a good linear fit for higher concentration of sucrose i.e 50g/l and above. For lower concentrations the results were unsatisfactory. From the Table 1.4 it can be seen that the values of α. are higher than β, hence it can be inferred that the contribution from the α growth associated parameter is more than, β non growth associated parameter, hence the product formation kinetics follows growth associated pattern. This model has one major drawback, despite the advantages of its simplicity i.e., product formation appears to be independent of the substrate concentration. Hence further studies are needed to afford better mathematical expression for product formation.



Fitting of the logistic model to experimental data for biomass concentration in batch cultivation

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Fig.1.7 (a) Initial sucrose concentration 10g/l. (R^2=0.541)

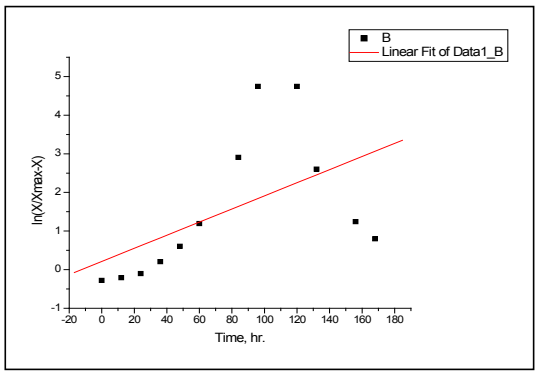


Fig. 1.7 (b) Initial sucrose concentration 20g/l (R^2=0.533)

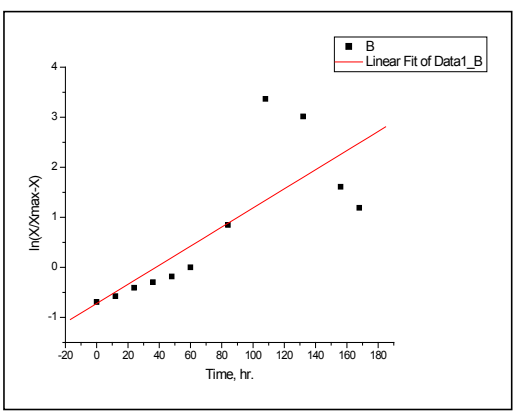


Fig. 1.7 (c) Initial sucrose concentration 30g/l (R^2=0.778)

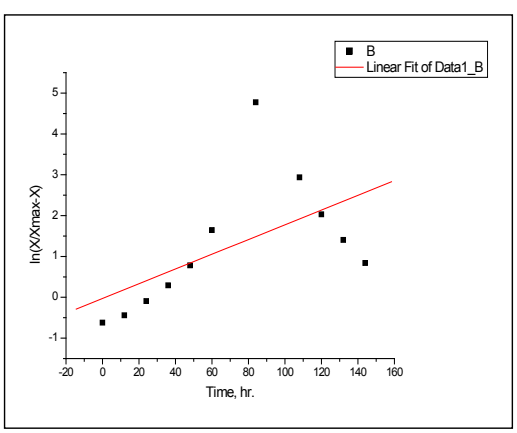


Fig. 1.7 (d) Initial sucrose concentration 40g/l (R^2=0.571)

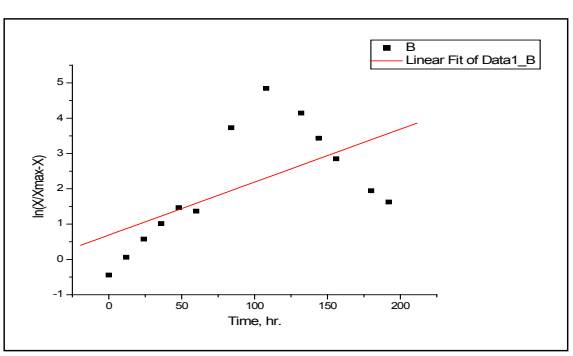


Fig. 1.7 (e) Initial sucrose concentration 50g/l (R^2=0.605)

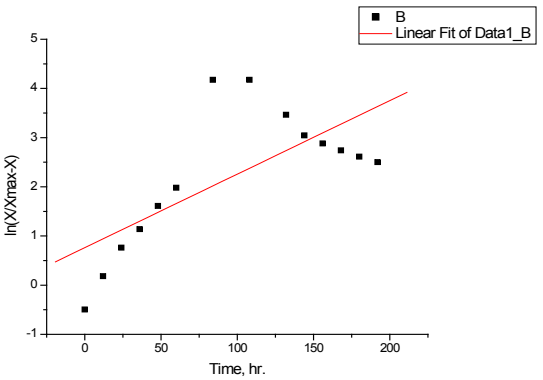
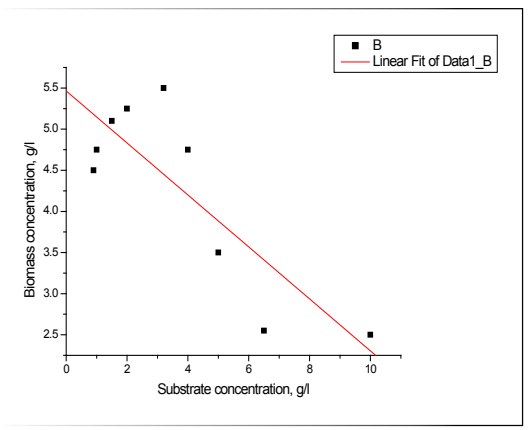


Fig. 1.7 (f) Initial sucrose concentration 60g/l. (R^2=0.703)



Yield factor for cell mass (Yx/s) from batcch cultivation studies.

Fig. 1.8 (a) Initial sucrose concentration 10g/l. (R^2=0.835)

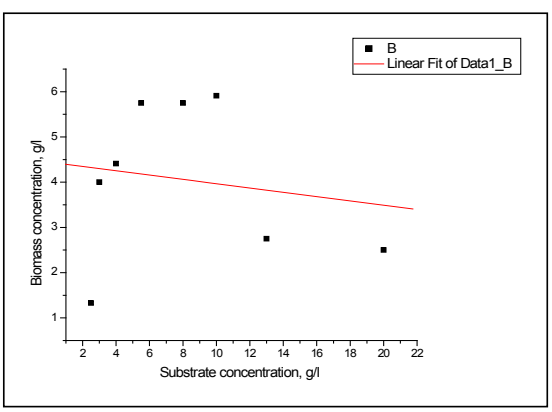


Fig. 1.8 (b) Initial sucrose concentration of 20g/l (R^2=0.164)

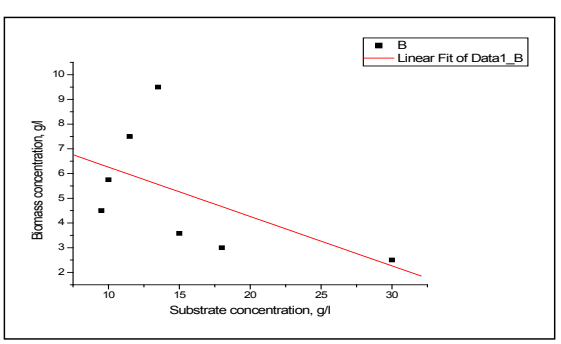


Fig. 1.8 (c) Initial sucrose concentration of 30g/l. (R^2=0.554)

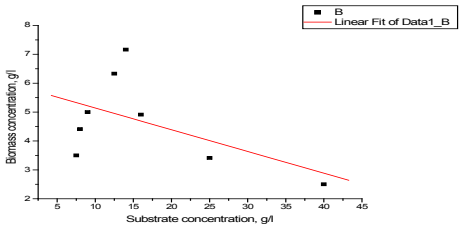


Fig. 1.8 (d) Initial sucrose concentration of 40g/l. (R^2=0.536)

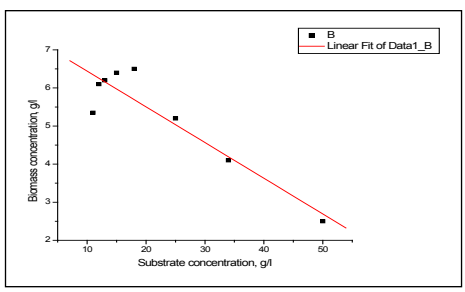


Fig. 1.8 (e) Initial sucrose concentration of 50 g/l. (R^2=0.925)

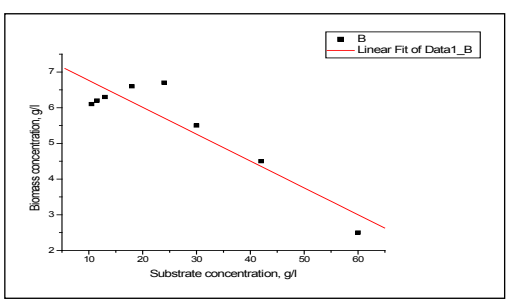


Fig. 1.8 (f) Initial sucrose concentration of 60g/l. (R^2=0.920)

μ_{max} calculated from logistic model

Initial sucrose concentration (g/l)	μ_{max} (per hour)
10	0.010
20	0.016
30	0.019
40	0.018
50	0.015
60	0.014

Table 1.3 Biomass yield coefficient (Yx/s) obtained for different initial sucrose concentration from the plots of biomass concentration Vs substrate concentration (from fig 1.8 (a) to 1.8 (f).

Initial sucrose concentration (g/l)	Y x/s (g dry wt. of cells/g of substrate)
10	0.31
20	0.04
30	0.20
40	0.07
50	0.09
60	0.07

Table 1.4 α and β (From 1.9 (a) to 1.9 (f)) of Leudeking-Piret model.

Initial sucrose concentration (g/l)	α	β
10	0.47	0.019
20	0.135	0.038
30	0.67	0.029
40	0.956	0.027
50	6.107	0.028
60	4.57	0.010

Variation of specific product formation rate with the specific growth rate.

Fig. 1.9 (a) Initial sucrose concentration of 10g/l. (R^2=0.119)

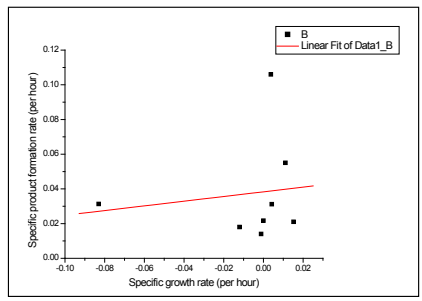


Fig. 1.9 (b) Initial sucrose concentration of 20g/l. (R^2=0.139)

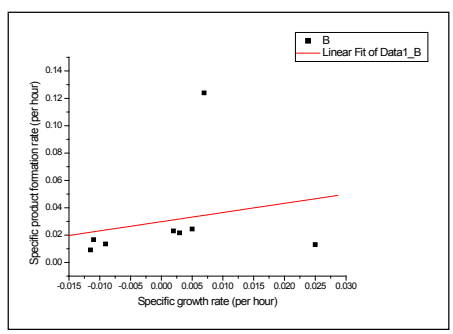


Fig. 1.9 (c) Initial sucrose concentration of 30g/l. (R^2=0.213)

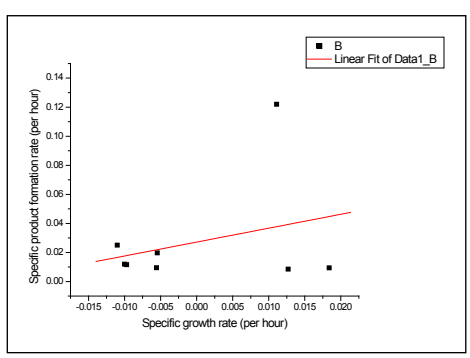


Fig. 1.9 (d) Initial sucrose concentration of 40g/l. (R^2=0.295)

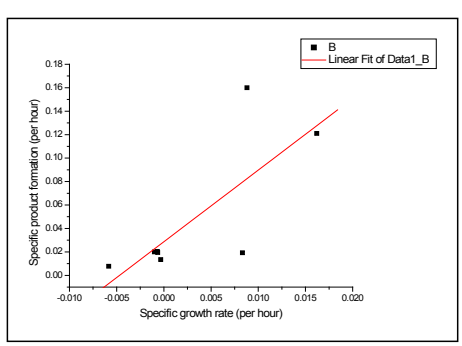


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Fig. 1.9 (e) Initial sucrose concentration of 50g/l. (R^2=0.758)

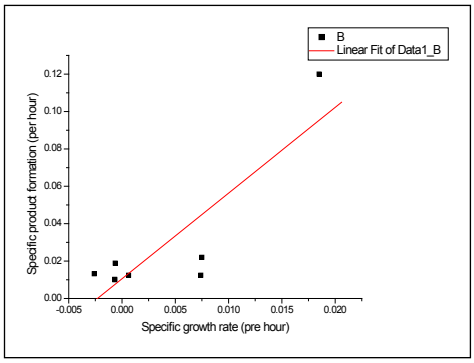

Fig. 1.9 (f) Initial sucrose concentration of 60g/l. (R^2=0.864)

Conclusions:

In the present study with sucrose as sole carbon source about 50g/l, was found to be optimum concentration, at which 29mg/l of trehalo-lipid was synthesized. The minimum trehalo-lipid production was observed to be at initial sucrose concentration of 10g/l. Logistic model for biomass growth was found to be very suitable for higher range of initial substrate concentrations, but it fails to predict for lower range of substrate concentration. Hence to validate the results, further study is needed, over wide range of initial substrate concentration. The maximum specific growth rate μ_{max} estimated usinglogistic model was 0.0153h^{-1}. The yield coefficient was found to be $Y_{x/s}$=0.13g dry wt. of cells/g of substrate. Leudeking-Piret model for production formation kinetics predicts well for higher initial concentration that is for 50g/l and more, but fails to predict for lower range of substrate concentration. From the application of Leudeking-Piret model it was found that the values of **α** (growth associated parameter) were much higher than **β** (non-growth associated parameter). From the higher values of **α,** it can be inferred that product formation kinetics is more of growth associated than non-growth. Although Leudeking-Piret model predicts well for product formation kinetics in the present study, this model has one major drawback, despite its advantage of its simplicity, the product formation appears to be independent of substrate concentration. Hence, further studies are needed to afford a better mathematical expression for product formation.

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