To Study and Analysis of Continuous Stirred Tank Reactor (CSTR) for Metabolites Production

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Abstract: Fermentation is very important biological processes for metabolite production; utilizes plant, animal or any microbial cells. These cells have been added in production media at laboratory or industrial scale to convert the substrates into our main metabolites production with combination with byproducts. Byproducts concentration can be produced more if biological cells growth conditions are not optimized. Continuous Stirred Tank Reactor (CSTR) is one of mode of fermentation which is applied in many metabolites production. This mode is very useful when we use controlled condition and maintained steady state culture during period time for continuous metabolite production. We use fermentation medium for supply appropriate energy for growth of microorganisms and product formation in fermentation process. A lot of carbon and nitrogen substrates combination with micro or micronutrients are also supplied to culture media for growth of microorganisms to produce the various metabolites such as simple sugar, organic acids or biofuel etc. Various metabolic pathways are found in various biological cells to involve produce the specific metabolite and enzymes of pathways are deciding the efficiency of metabolite, generated in biological cells.

Key words: *Fermentation, Biological cells, metabolites, CSTR, concentration, growth conditions*

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I. Introduction

The fermentation medium must supply appropriate energy for growth and product formation and must meet the elemental and specific nutrient requirements of the organism. A remarkable increase in citric acid volumetric productivity was reported in continuous citric acid fermentation in a supported biomass reactor via immobilized-mycelium system (Sommariva et al., 1997). It is also reported that continuous fermentations in CSTR could allow remarkable productivity increases with respect to both submerged and surface batch processes, even if large scale industrial applications are not forseeable in the present future (Royhr et al. 1983).

It is reported that concentrations of 7 medium components were optimized automatically within 40 continuous experiments to result in a maximum growth rate of the methylotrophic yeast *Candida boidinii* and growth-linked production of the formate dehydrogenase enzyme (FDH) (Beste et al., 1997). The batch or fed-batch operations were feasible modes of optimal operation and the CSTR was analyzed with steady state analysis separately from that of dynamic operation. It was possible to determine the feasible modes of optimal operation with nonsingular transformation without checking all the cases of possible operation (Lee, 1999).

Modak and Lim developed an application of singular control algorithm to continuous stirred tank reactor (CSTR). They applied the singular control application to various control modes and determined feasible operational modes. They proposed that there is no singular control when there is no inlet feed. The singular control of outlet feed rate is only possible when the inlet feed rate is at the singular state (Modak and Lim, 1992). It was reported that in batch reactor, maximum reaction can be maintained for short period and microbial environment was constantly changing.

The major drawbacks of performing medium optimization with batch experiments in shake-flasks were the impossibility to maintain a constant pH over a prolonged period of time, the low oxygen transfer rate due to the surface aeration, and the reduced reproducibility of the batch experiments caused by alterations in the quality of the seed culture (Yee and Blanch 1993). CSTR is a better solution which eliminates this restriction and provides the unchanging microbial environment. D-ribose has been reported to be beneficial in some rare genetic diseases, such as adenylosuccinase deficiency and myoadenylade deaminase deficiency (De Wulf and Vandamme 1997c). D-ribose is used as raw material in synthesis of riboflavin (vitamin B2) and nucleotide flavors enhancers in pharmaceutical industry. Here author will show results and discussion of D-ribose sugar which is produced by batch mode as well CSTR mode. But we focus here CSTR modes only.

Medium and chemical Compositions:

For D-ribose production, transketolase deficient mutant strain *B. pumilus* was cultivated in different media. The strain was maintained on LB agar slant and was stored at

4^0C. Fresh slants were prepared every three months from the stock by growing the cells in LB broth medium

Table: Description of three different medium compositions used in cultivation of *B. pumilus* at different stages. Medium1 was used as first stage of strain cultivation and cells were picked from storage LB agar slants. Aim of cultivation of strain in medium1 is to obtain the active inoculum for seed medium. Medium 2 is used as seed medium and inoculated with cells of medium 1. In medium 2, inoculum for production medium (medium3) is prepared. The detail of each medium is shown in this table (Srivastava1 and Wangikar., 2008;).

Growth medium (grams per liter of distilled water)	**Medium 1** (Pre-seed)	**Medium 2** (Seed medium)	**Medium 3** (Production medium)
	25.0 g of Luria Bertani (LB) broth	D-Sorbitol, 20 g; Corn Steep Liquor, 20 g; K_2HPO_4, 3.0; KH_2PO_4, 1.0 g.	D-glucose, 25- 250 g; Ammonium sulfate (AMS), 0 - 10 g; $CaCO_3$; 16 g, $MnSO_4$; 0.5 g, L-leucine; 0.5 g, L-tryptophan; 0.05 g, Supplementary substrate; Casamino acid (0-20 g), Corn steep liquor; 0-20 g, All twenty amino acids (6.5mM) KH_2PO_4 (0-1.0 g), a group of four amino acids (1.5 g) each

Chemical Compositions

Chemicals used throughout this work were laboratory reagent grade unless otherwise indicated. Luria-Bertani (LB) broth, D-glucose, standard D-ribose, L-tryptophan and L-leucine were procured from Hi-Media (Mumbai, India) whereas KNO_3, $(NH_4)_2SO_4$, KH_2PO_4, $MnSO_4$, Ethanol, NaOH and $CaCO_3$ were procured from Merck (New Jersey, USA). HCl, Acetic acid, Sodium acetate, DMSO, Trichloro acetic acid, Sodium thiosulphate, NaCl, amino acids, Ortho-phosphoric acid and Ninhydrin are products from the S.D.Fine chemicals, Boiser, India. Corn steep liquor (CSL) solids, Hydrindantin and Azocasein were purchased from Sigma (St. Louis, MO, USA) and casamino acids were purchased from Becton Dickinson and Co. (Sparks, MD, USA). D-sorbitol is product from Thomas Baker Laboratories, Mumbai, India.

II. Analytical methods:

Spectrophotometer detection

Dry cell weight was determined by taking different amount of cells in eppendorf tubes (empty tube weighted). The broth was centrifuged at 8000 rpm for 5min. Biomass was washed twice with the volume of distilled water. Then, biomass was dried in microwave (Ken-star) oven at higher temperature for appropriate periods. Biomass was weighted. OD of cells was also determined by taking OD at 600nm.

High Performance Liquid Chromatography (HPLC) method: For D-glucose and all metabolites:

2 ml of fermentation sample was taken and centrifuged at 10,000rpm for 10 minutes and the resulting supernatant was filtered through 0.45µm pore size filter paper. 20 µL of the filtrate was injected into the HPLC (Srivastava et al., 2009).

III. Results:

D-ribose production was studied in a CSTR. Different biomass formation rates were observed for different glucose consumption rates in the course of fermentation processes with dilution rate varying from 0.022 h^{-1} to 0.044h^{-1}. Normally in production media, maximum specific growth rate of mutant strain *B. pumilus* in batch mode was found in range of 0.1h^{-1} -0.35 h^{-1} depending on medium compositions. The specific glucose uptake rates for specific D-ribose formation rate were studied at steady state in different production mediums. The objective of the work was to produce the D-ribose via continuous mode of fermentation. So we have been succeeded to produce D-ribose via CSTR.

CSTR mode:

One of the most important features of chemostats is that micro-organisms can be grown in a physiological steady state (µ=D). In steady state, all culture parameters remain constant (culture volume, dissolved oxygen concentration, nutrient and product concentrations, pH, cell density, etc.). Micro-organisms grown in chemostats naturally strive to steady state: if a low amount of cells are present in the bioreactor, the cells can grow at growth rates higher than the dilution rate, as growth isn't limited by the addition of the limiting nutrient. The limiting nutrient is a nutrient essential for growth, present in the media at a limiting concentration (all other nutrients are usually supplied in surplus). However, if the cell concentration becomes too high, the amount of cells that are removed from the reactor cannot be replenished by growth as the addition of the limiting nutrient is insufficient.

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Study on defined medium containing glucose with AMS:
Mutant *B. pumilus* was grown on defined medium and fermentation studies were conducted in CSTR. The medium contained D-glucose (100 $g.l^{-1}$) as carbon source and AMS (5 $g.l^{-1}$) as nitrogen substrate. Low biomass formation was observed. The reactor was initiated in batch mode and then it was switched to CSTR mode after 37h at a dilution rate of $0.022h^{-1}$. CSTR operation was maintained up to 127 h. After that the dilution rate was changed from 0.022 h^{-1} to 0.044 h^{-1}. At this new dilution rate the cell growth rate decreased and eventually washout occurred at 155h. No D-ribose formation was observed in this medium possibly due to low specific glucose uptake rate at steady state (i.e., between 95-127 h). To understand the glucose consumption, biomass and D-ribose formation pattern the experiment was run in batch mode up to 37 h in this medium. Glucose consumption rate with biomass formation rate was monitored by analyzing samples collected at an interval of 6 or 12 h. In batch mode, biomass concentration in this medium was found to be low and it was around 2.3 $g.l^{-1}$ (i.e. highest concentration) due reaching of cells at exponentially stage (37 h). It was observed that initial phase of CSTR mode operation helped to consume D-glucose without D-ribose formation in fermentation broth and simultaneously biomass formation was observed to gradually decrease with time (figure 7.1 A). At steady state, specific glucose uptake rate was found in range of 0.08-0.09 $mM.g^{-1}h^{-1}$ which was too low for D-ribose formation. And the stir rate (rpm) was found in range of 375-500 rpm to maintain the DO level at 40%. Biomass concentration was in the range of 1.0-1.2 $g.l^{-1}$. Due to lack any complex nitrogen source, this media didn't favor higher biomass formation and this is why the specific glucose uptake rate was found lower (figure 7.1 B). Only two byproduct formation was observed in this medium in varying concentration. These byproducts were acetic acid (0-0.3 $g.l^{-1}$) and acetoin (1.8-3.7 $g.l^{-1}$) in fermentation broth (figure 7.1 C). In this medium we have noticed that acetic formation is much lower comparison to medium containing complex nitrogen source medium.

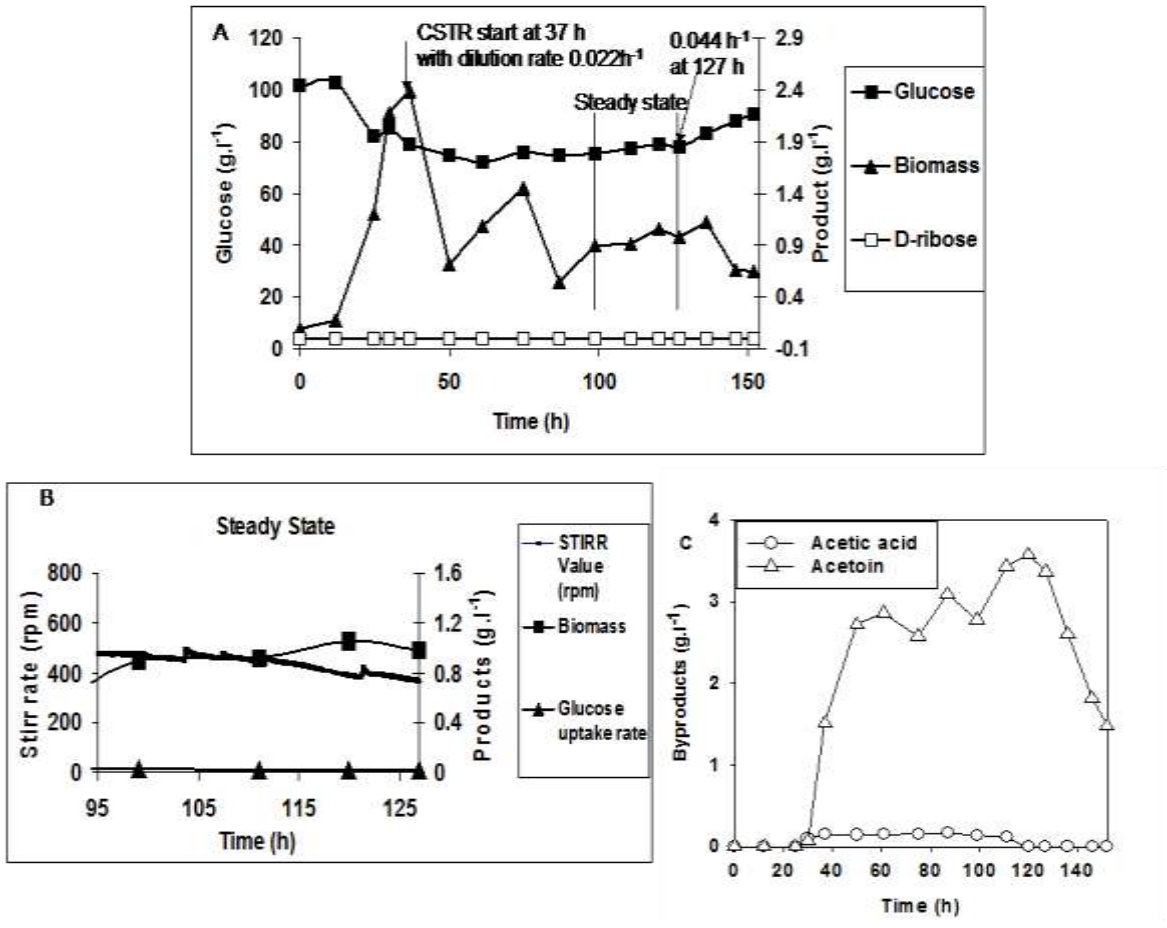

Figure 1 A Offline profiles in defined production medium in CSTR mode. (A) Glucose, biomass and D-ribose concentrations; (B) glucose uptake rate, biomass concentration with stir rate at steady state (C) Byproduct concentrations. Medium composition are; glucose, 100 g; AMS, 5.0g; other media compositions was: $CaCO_3$, 16 g; $MnSO_4$, 0.5 g; L-leucine, 0.5 g; and L-tryptophan, 0.05g Base solution (2M) was added to maintain the pH in fermentation broth and it was seen that less amount of base solution (i.e. 125ml) was used to maintain pH. It was found that there is no pH increase beyond 6.5 in growth phase of cells (figure 1A). At

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steady state, pH (6.5) was maintained by base addition (amount 20-30 ml). Stir rate (rpm) was kept in cascade mode in range of 200-700 rpm to maintain DO level (i.e. 40%) (figure 1 B).

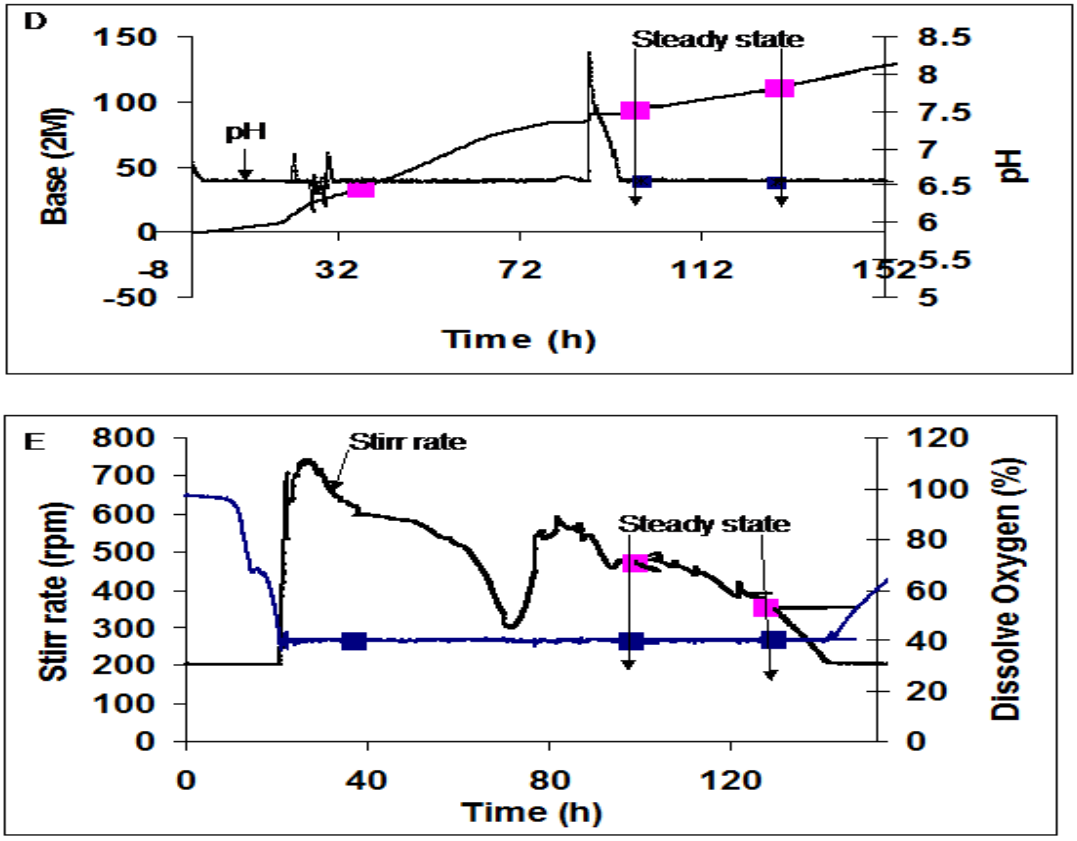

Figure 1B Online profiles of D-ribose production in defined production medium in CSTR mode. (A) pH profile with base addition (B) Dissolve oxygen with stir rate. We have maintained pH at 6.5 by addition of 2 M NaOH solution in CSTR experiments and it is seen that 130 ml of NaOH solution was come to maintain pH. In subfigure 7.2 B, stir rate was set up in cascade mode to maintain the dissolved oxygen (40%). Refer to figure 7.1 to legend for medium composition.

Discussion:

D-ribose production by *tkt* deficient *Bacillus pumilus* strain in batch and fed-batch fermentation has been widely reported in literature. To the best of our knowledge this is the first study reported on production of D-ribose via Continuous Stirred Tank Reactor mode. Two different production mediums were used to cultivate the cells. Some interesting observations were made during the CSTR mode of operation. Glucose consumption rate might be related to the presence of complex nitrogen substrates in production medium. The first medium was defined production medium (containing 100g glucose and 5.0 g AMS) without any complex or organic nitrogen substrate. In this medium, there was no D-ribose production during course of fermentation (~batch or CSTR mode). It is might be due less biomass formation, less glucose consumption rate or specific glucose consumption rate at steady state. At $0.044h^{-1}$ dilution rate, growth cells were found very less and after certain time washout phenomena were occurred. In this dilution rate, maximum specific growth was around $0.001h^{-1}$. In the second production medium that contained complex nitrogen substrate along with higher glucose (163 g) and ammonium sulfate (9.6g), higher biomass formation and D-ribose production was observed. In the second medium it was also observed that at steady state specific glucose uptake rate and D-ribose formation rate was much higher compared to defined production medium. It is clearly visible from figure 1B that there was no D-ribose formation in defined medium in the entire batch. Two different dilution rates were used in CSTR mode of operation and washout was observed when dilution rate changed. We have performed three CSTR run in defined media but this did not lead to D-ribose production. We have found that cell growth formation is started in longer time and concentration was also less. This is due to no complex nitrogen substrate. It is noticed that glucose consumption was found very less which was not sufficient for D-ribose formation. Then we have design the production media which contained two complex nitrogen substrates. On this basis, a second CSTR run was designed with medium containing higher glucose and complex

nitrogen substrates along with higher AMS. Single dilution rate of .022h^{-1} was used throughout the run (figure 1A). In other experiment, we have noticed the low D-ribose level at late period of CSTR experiment (that was due to wash out cell concentration with time in CSTR mode).

IV. Conclusions

Fermentation is found a suitable biological process for metabolite production such as citric acid as organic acid, low weight sugar such as D-ribose or biofuel production. In these productions, varieties of carbon and nitrogen substrates with suitable microbe's culture are utilized. Fermentation are performed in batch, fed-batch or continuous modes (e.g. CSTR) and modes will decide with metabolite nature and culture growth condition. We have maintained specific dilution rate for medium during production which could not permit to washout phenomena in culture. Glucose is very preferential substrate for most of microbes or any other biological cells. They have to utilize best way to maintain the optimum physiological condition to produce the larger quantity of specific metabolite in specific cell culture.

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