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STUDIES ON THE EFFECTS OF END PRODUCT INHIBITION OVER LACTIC ACID BACTERIA UNDER HIGH CELL DENSITY CULTIVATION PROCESS

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ABSTRACT

Lactic acid bacteria (LAB) have recently caught the rapt attention of medical and scientific researchers as they form the part of gut microflora and affect the host beneficially as probiotic organisms. Lactic acid is the major metabolic end-product of LAB culture through carbohydrate fermentation, which finds its potential use in food, chemical & pharmaceutical industry. Reduced biomass concentration is the major bottle- neck in the use of LAB culture for the production of lactic acid due to end product inhibition. It becomes inevitable to search an alternate fermentation process towards attaining high levels of biomass accumulation. Two strains of Lactic acid bacteria namely *Lactococcus lactis*, *Lactobacillus reuteri*, had been employed for the production of lactic acid. The growth kinetic parameters such as specific growth rate, doubling time, oxygen requirement, substrate utilization profile and yield coefficient with respect to biomass formation were determined under optimal cultivation conditions. These cultures were experimented towards high cell density cultivation process which yielded a remarkable increase in the product as well as better viability of the cell. By employing the total cell retention culture technique the biomass concentration has been found increased up to 50.19%, 51.75% respectively. On these test cultures, the effects of lactic acid inhibition in terms of the minimum inhibitory concentrations, bacteriostatic and bacteriocidal concentrations were also been determined. An overall decrease in viability and significant changes in the morphological structures concludes that *L. reuteri* is less susceptible than *L. lactis*. The attempts towards strain improvement and effect of bio-parameters on lactic acid tolerance are currently under investigation. Based on this improved alternate process, the Lactic acid bacterial cultures can be well employed for important industrial purposes as this work provides a scope for overcoming the product inhibition effects.

Key words: Probiotic, Lactic acid bacteria, End product inhibition, Total cell retention technique, High cell density culture.

INTRODUCTION

Lactic acid, also known as milk acid is a major metabolic product of carbohydrate

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fermentation and finds its use in food industry, confectionery, beer and wine industry, dairy industry, cosmetic industry, chemical & pharmaceutical industry contributes to the organoleptic and textural profile of a food item as acidulant, preservative and pH regulator etc.

The Lactic Acid Bacteria (LAB) comprises of a clade of Gram-positive, low-GC, non-sporulating, micro-aerophilic rods or cocci that are associated by their common metabolic and physiological characteristics. These bacteria found in decomposing plants, produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Accumulation of Lactic Acid inhibits the growth of Lactic Acid Bacteria. This end product inhibition is due to alteration of pH in to more acidic which in turn affects the growth of the cells and reduction in the viability.

Large scale production of microbial products is possible by adopting high cell density cultivation strategy is used to improve microbial biomass and product formation with high volumetric productivity, reduced product inhibitory effects and reduced culture volume which makes downstream processing easier.[1] Fed batch culture techniques have often been used to achieve high cell density. Batch culture fermentation was used to produce a maximum biomass of *L. reuteri*.[2]

In the industrial production of lactic acid, the biomass increase is the major bottle neck because of the end product inhibition. Hence, the current research has been conducted in quest for searching an alternate culturing process that would satisfy the biomass build up aimed towards rendering solution for the important industrial applications. Total cell retention culture technique had been adapted to result in relatively higher biomass concentration than that was obtained during the predetermined growth kinetics for the same culture. End product inhibition studies were also been conducted and the minimum inhibitory concentration was ascertained.

EXPERIMENTAL

Materials and methods

Lactic acid bacteria

*Lactococcus lactis (*NZ 9000) is an AT-rich, Gram-positive bacteria used extensively in the production of buttermilk and cheese. They are cocci that group in pairs and short chains, typically 0.5-1.5 µm in length, non-sporulating and non-motile.

Lactobacillus reuteri (ATCC 55730) is a Gram-positive bacterium that naturally inhabits the gut of mammals and birds. Their growth is optimum at pH 5.5-5.8 and the organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals etc.

Culture medium

MRS Broth: This medium is specific for the isolation and enumeration of Lactobacilli sp. Composition (g/L): Peptone from casein 10.0; Yeast extract 5.0; Beef extract 10.0; D (+) glucose 20.0; potassium dihydrogen phosphate 6.0; ammonium citrate 2.0; Tween®80 -1.0; sodium

acetate 5.0; magnesium sulphate 0.575; iron (II) sulphate 0.034; manganese sulphate 0.12; agar-agar-15.0 the pH was adjusted to 5.5. The microbial cultures were maintained in *Rogosa agar* plats and slants at 4^{o}C.

Analysis of microbial growth

1% inoculum in 200 mL of MRS broth was taken under shake flask cultivation method to study the growth kinetics of *Lactic acid bacteria* and incubated at 37^{o}C. The periodical sterile samplings were done for every 30 minutes with a sample volume of 3 mL. The culture was analyzed for its absorbance at 600 nm with respect to time (Apella, M. C. et al., 1992). The Specific growth rate (μ) Doubling time (t_d) and Dry cell weight of the culture were calculated.

Residual substrate estimation

Glucose on oxidation in the presence of glucose oxidase (GOD) produces D-Gluconic acid and hydrogen peroxide. Hydrogen peroxide is oxidized in the presence of Peroxidase (POD) which is used for converting phenol and 4 amino antipyrine to quinoneimine results in development red colour. The colour change is detected at 505 nm. By employing above GOD / POD test assay procedure, total residual glucose content was estimated to calculate Yield coefficient ($Y_{x/s}$) with reference to biomass formation.

Total cell retention culturing process

The microbial culture were grown under optimal conditions for 12 hrs and harvested by centrifugation at 7000 rpm for 10 minutes at 4^{o}C under sterile conditions. The biomass pellet obtained was resuspended into fresh media that contained 1.5 times more glucose than the original media. The same procedure of growth and biomass harvest were repeated 3 times to gain more amount of biomass build-up. The growth kinetics was monitored by taking periodical sampling.

End product susceptibility test

Micro-dilution test was performed to find the Minimum Inhibitory Concentration (MIC) of lactic acid on *Lactobacillus sp.* The inoculums were allowed to grow up to exponential phase[3] and test was conducted as per the scheme shown in the Table 1. The experimental cultures were incubated in shaker at 37^{o}C overnight.

Table 1: Micro-dilution test for end product inhibition analysis

Tube No.	Control	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
Volume of media (mL)	2	2	2	2	2	2	2	2	2	2	2
Inoculum volume (μL)	200	200	200	200	200	200	200	200	200	200	200
Conc. of lactic acid (μg/mL)	-	22.5	45	67.5	90	112.5	135	157.5	180	202.5	225

The culture tubes were examined after every 2 hrs for its growth by absorbance at 600nm using respective blanks and number of colony forming units was calculated by plating method. The minimum Inhibitory concentration value (MIC) was calculated. The microscopic examination was done intermittently to check the effect of lactic acid on the morphology of cells.

RESULTS AND DISCUSSION

Determination of effect of agitation on growth

The test cultures were checked for its growth under static and under agitation at 150 rpm. The results are tabulated in Table 2.

Table 2: Effect of agitation on growth

Microbial culture	Static (μ min^{-1})	Agitation (μ min^{-1})	Doubling time (t_d)
Lactococcus lactis	0.00475	0.00482	145.9 minutes (under agitation)
Lactobacillus reuteri	0.00522	0.00155	132.5 minutes (under static)

There is 70.3 % more specific growth rate is found in the case of *L. reuteri* culture maintained at static when compared with at 150 rpm. This implies that a very limited oxygen concentration required i.e. micro-aerophilic, where as *L. Lactis* cultures shows aerobic nature. Further studies had been conducted, under static condition for *L. reuteri* and under agitated condition for *L. Lactis.*

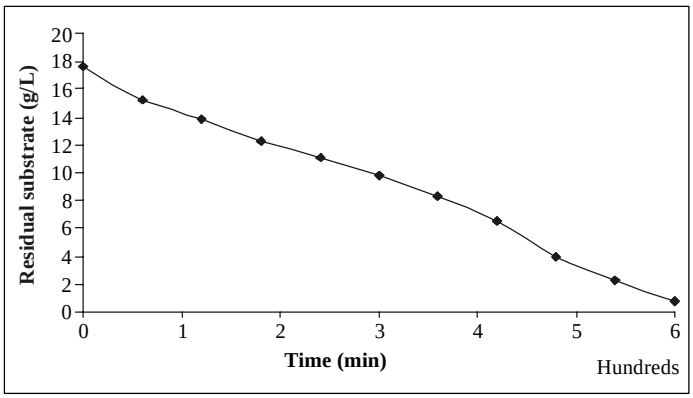

Fig. 1: Substrate consumption profile of *L. lactis*

Substrate utilization profile and yield coefficient analysis

From the above graphs it is evident that *L.lactis* and *L.reuteri* cultures are in active logarithmic phase of growth by utilizing substrate 96.68% & 97.19%, respectively. There is an

overall raise in yield co-efficient with respect to the biomass formation, which implies that the added substrate concentration is fully utilized by the organism only for building up the biomass concentration and not towards producing any metabolites.

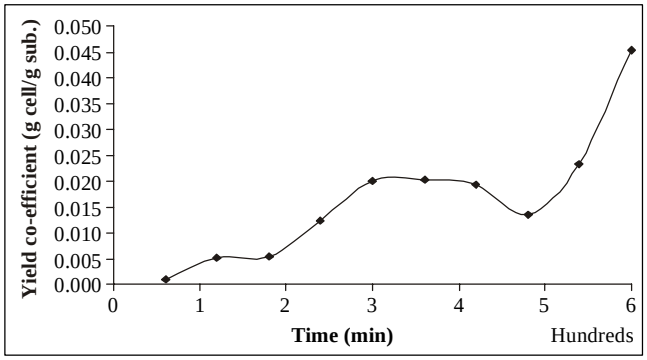

Fig: 2 Yield coefficient profile of *L.lactis*

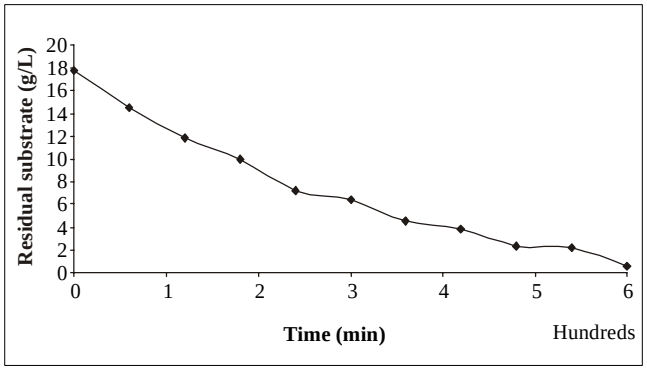

Fig: 3 Substrate consumption profile of *L.reuteri*

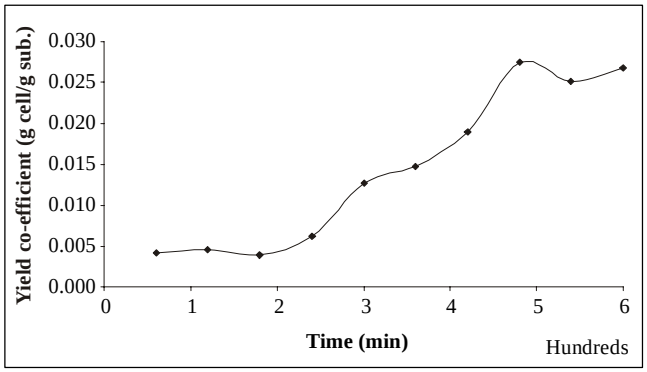

Fig: 4 Yield coefficient profile of *L.reuteri*

Biomass Accumulation under total cell retention culture process

There is a linear increase in the biomass concentration observed in L. lactis & L. reuteri under TCRC process up to 0.632 g/L, 0.713g/L, respectively.

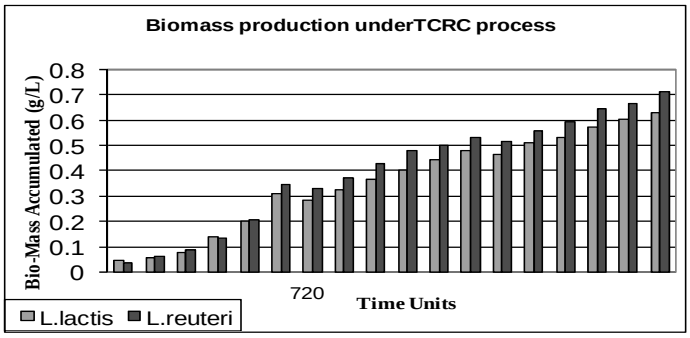

Fig. 5: Biomass accumulation profile of *L. lactis & L. reuteri* under TCRC process

Even though there is a increase in overall biomass concentration, the specific growth rate at the given time is found to be decreasing with increase of substrate concentration (Table 3).

Table 3: Effect of substrate concentration on growth profile

Substrate conc.	*L.lactis* (μ min^{-1})	*L.reuteri* (μ min^{-1})
20 g/L	0.00475	0.00522
50 g/L	0.00208	0.00206
80 g/L	0.00183	0.00199

Both the cultures show similar trend of yield coefficient with reference to biomass concentration during the first phase of cell cultivation (Figs. 6 and 7) i.e. *L. reuteri* shows an increase of 57.34 % over *L. lactis* with could be due its better growth rate under micro-aerophilic condition. The same trend could be seen in second phase of cell cultivation. During third phase of cell cultivation with 80 g/L of substrate concentration, *L. lactis* shows 51.7 % higher yield over *L. reuteri*. This proves that *L. lactis* strain is less susceptible with excess substrate inhibition when compared to *L. reuteri.*

End Product Inhibition analysis

Lactic Acid when added in incremental concentration from 22.5 µg/mL to 225 µg/mL as per micro-dilution test scheme (Table 1), there is 72.72 % and 84.61% of reduction in overall biomass concentration been observed in the case of *L.lactis* & *L. Reuteri* respectively. In the case of *L. lactis* (Fig. 8) lactic acid concentration up to 135 µg/mL found to have bacteriostatic inhibitory effect and at 157.5 µg/mL found to have bacteriocidal inhibitory effect. However in the case of *L.reuteri* (Fig. 9) even though there is no remarkable decrease in biomass initially, upon 6 hrs of incubation, there is a uniform bacteriocidal inhibitory effect in the all

concentrations of lactic acid. Remarkably in the case of *L. reuteri* there were changes in morphological structure as the cells were clumped together into clusters and were found to be elongated than the normal size, which could be an indication that the cells are under stress due to the end product inhibition. From the above trends it has been concluded that *L. reuteri* is less susceptible than *L. lactis.*

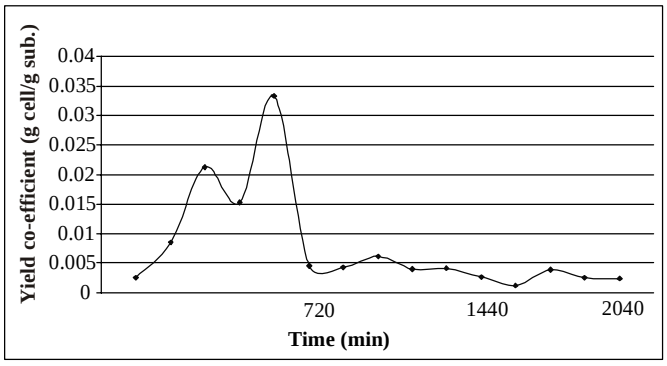

Fig: 6 Yield coefficient profile of *L lactis* (TCRC)

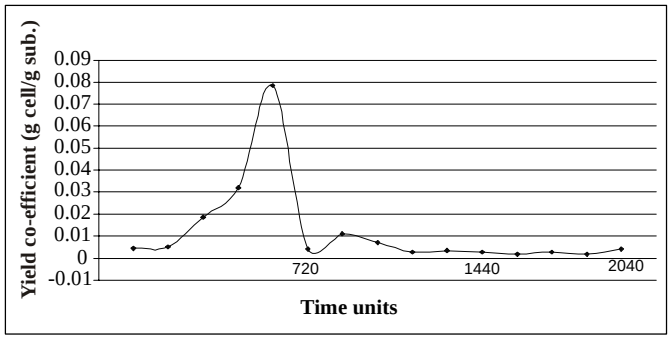

Fig: 7 Yield coefficient profile of *L.reuteri* (TCRC)

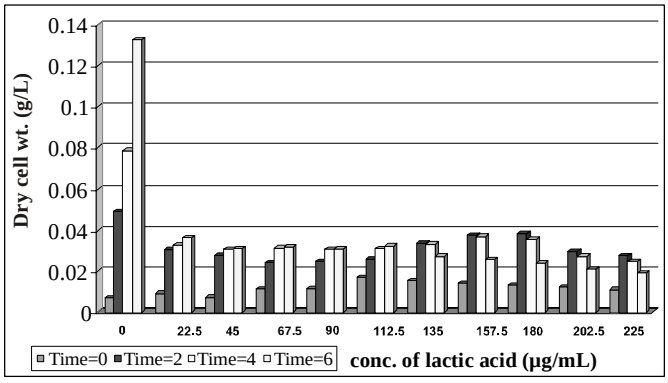

Fig. 8: End product inhibition profile of *L. lactis*

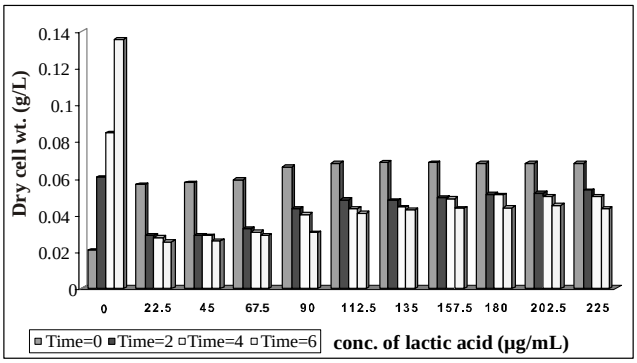

Fig. 9: End product inhibition profile of *L. reuteri*

CONCLUSION

The lactic acid bacteria produce lactic acid as the major metabolic end-product of carbohydrate fermentation. The biomass build up is the major bottle neck in the use of LAB for the production of Lactic Acid due to end product inhibition and feedback inhibition. As lactic acid finds its potential use in dairy industry, beer and wine industry, cosmetic industry, chemical & pharmaceutical industry, there is a need in search of alternate culturing process to yield higher cell density of LAB. Two strains of Lactic acid bacteria namely *Lactococcus lactis, Lactobacillus reuteri* had been employed for analysing the growth kinetic parameters such as Specific growth rate, Doubling time, Oxygen requirement, Substrate utilization profile and Yield coefficient with respect to biomass formation under optimal cultivation conditions. By employing the Total Cell Retention Culture technique the biomass concentration has been found increased remarkably. Thus this method could be suggested as the best alternate method in order to increase high cell density of lactic acid cultures. On the basis of results of end product inhibition over these cultures, it is concluded that *L. reuteri* is less susceptible than *L. lactis.* These cultures can be comfortably used for important industrial purposes as this work provides a scope for overcoming the product inhibition effects.

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