Comparative Batch Growth Studies of Pure *Lactobacillus* Strains and Their Co-culture in Synthetic Medium with Different Neutralizing Agents

Manoj K. Ghosh[1], U.K. Ghosh[2]
Department of Paper Technology, Indian Institute of Technology Roorkee,
Saharanpur Campus, Saharanpur-247001, India
E-mail: [1]mkengg2004@rediffmail.com, [2]ghoshuk_iitr@yahoo.com

Lactobacillus strains have immense potential of being utilized as multifunctional microorganism used in various chemical, biochemical, food, pharmaceutical and dairy industries. Lactic acid (2-hydroxy propanoic acid) synthesized by different *Lactobacillus* strains serves as feed stock for various industries. To suppress the inhibitory effects of free lactic acid on microbial growth and synthesis, during fermentative production of lactic acid, the free lactic acid has to be converted to lactate form by employing neutralizers such as NaOH, $CaCO_3$, etc. In the earlier investigations, inhibitory effects of calcium carbonate towards growth and production and the specific role of calcium ions in phage infection of *Lactobacillus* have been reported. Therefore, in the present studies treatment of *Lactobacillus* with NaOH has been compared with that of $CaCO_3$ which is commonly used neutralizer in lactic acid fermentation. In present experimental investigation, studies on anaerobic batch fermentation with *Lactobacillus delbrueckii* (NCIM 2025), *L. pentosus* (NCIM 2912) and their co-culture have been carried out using glucose based synthetic media at 37°C, 180 rpm, initial pH 6.5 with pH maintained at 5.6 using either NaOH or $CaCO_3$ as neutralizer every 12 h. The first set of experiments with NaOH as neutralizer showed highest cell dry weight for *L. delbrueckii, L. pentosus* and their co-culture as 6.69, 11.51 and 23.95 g/l in 24, 24 and 36 h, respectively, while lowest pH values were 4.70, 4.55 and 4.50 attained in 48, 36 and 48 h, respectively. However, in the second set of experiments with $CaCO_3$ as neutralizer, the highest cell dry weights for *L. delbrueckii*, *L. pentosus* and their co-culture were observed as 9.09, 12.89 and 16.08 g/l, respectively in 48 h, while lowest pH values were 4.80, 4.70 and 4.88 attained in 48, 48 and 12 h, respectively.

1. Introduction

Lactobacilli are potent producers of wide range of antagonistic primary and secondary metabolites such as, organic acids, diacetyl (flavouring agent), bacteriocins (nisin) and antibiotics (Ross et al. 2002). Todorov et al.(2007) reported that *Lactobacilli* are also in demand for probiotic properties, as they play an important role in stabilizing the intestinal microflora by checking the colonization of pathogenic microorganisms. *Lactobacillus sp*. synthesize lactic acid (2- hydroxy propanoic acid) as their major product, that finds application in food, pharmaceutical and cosmetic industries and has various other industrial applications such as feedstock in preparation of different

chemicals (acrylic acid, propylene glycol, acetaldehyde and 2,3 pentandione), in adhesive formulation, as detergent builders, as terminating agents in phenol formaldehyde resins. Esters of lactic acid (stearoyl-2-lactylate, glyceryl lactopalmitate and glyceryl lactostearate etc.) are used as emulsifying agents in baking foods (Narayanan et al.2004a). It also serves as raw material for the production of poly lactic acid (PLA) which is a ecofriendly biodegradable polymer (Calabia et al. 2006). Adsul et al. (2007) reported that approximately 90% of world wide lactic acid production is through microbial fermentation and the rest is through hydrolysis of lactonitrile. Lactic acid production through chemical synthesis provides racemic DL lactic acid while microbial fermentation gives steriospecific L(+), D(-) or DL lactic acids based on specific microbial strains used (Altaf et al. 2006). The separation of particular form of lactic acid isomer from the racemic mixture is difficult and involves costly chromatographic techniques, hence production of lactic acid by microbial fermentation serves as a better option (Narayanan et al.2004b). Presser et al. (1997) reported that organic acids such as lactic acid are inhibitory towards bacterial growth as they can chelate essential growth elements like iron , while its undissociated form is lipophilic that could enter the bacterial cell and cause greater inhibition than externally active strong mineral acid. It has been reported by Mirdamadi et al.(2002) that without pH control of the fermentation broth, yield of lactic acid decreases by 30 – 50%, hence neutralizing agents such as sodium hydroxide, calcium carbonate and ammonium hydroxide are usually added, for higher yield of lactic acid. Inhibitory effect of $CaCO_3$ has also been observed on growth and production of *Lactobacillus* strains and pellet formation in *Rhizopus oryzae*. Sodium and calcium ions play biologically significant roles in prokaryotic and eukaryotic cells. It is reported by Sherman et al.(2006) that calcium is present in lower concentration than the sodium ions in the cells, and the cells keep calcium ions at low level as at higher concentrations calcium ions can bind to proteins and alter their enzymatic properties.

Thus with an objective to determine the effect of neutralizing agents like NaOH and $CaCO_3$ on biomass growth (cell dry weight) and pH drop(acid formation) , the present study included batch experiments with *Lactobacilli* pure culture and co-culture in glucose based synthetic media at 180rpm, 37^0C, initial pH6.5 and with pH maintained at 5.60. The study also evaluates the compatibility of co-culture with the neutralizing agent applied in terms of biomass growth and acid formation with respect to the *Lactobacillus* pure cultures.

2. Background Information

A living cell has to perform work in order to maintain difference in the composition of its internal medium with respect to the external one. When the work is carried out for the movement of solutes across the cell membranes, active transport of solutes across the cell membranes takes place against their electrochemical potential gradient. But diffusion of solutes (if polar) through membranes takes place down the electrochemical potential gradient, aided by the carrier proteins that overcome the activation energy required by that solute to enter through the lipid portion of the membrane (Jennings, 1995). During the fermentative production of lactic acid the enhancement of lactic acid concentration is reflected in the decline of pH in the fermentation broth. The

undissociated form of lactic acid is a strong growth inhibitor which diffuses across the cell membrane. Various theories have been proposed (Pieterse et al., 2005) about inhibitory effects of lactic acid on bacterial growth such as (i) Cytosol acidification due to acid influx, (ii) Dissipation of membrane potential and (iii) Accumulation of anions intracellularly. Intracellular accumulation of lactate anions from the diffusion of lactic acid in media can cause loss in water activity and end product inhibition, that can impede the regeneration of NAD^+ for the *Lactobacilli* under the anaerobic conditions when their cells could not regenerate NAD^+ through NADH oxidase. Thus it is suitable for *Lactobacilli* to maintain a proton gradient across the cell membrane, possessing a higher intracellular pH, than that of extra cellular medium. The influx of undissociated form of lactic acid (present in fermentation broth) in the bacterial cells causes the acidification of cytoplasm and dissipation of proton gradient. The cell in response effects extrusion of protons to maintain the proton gradient through energy dependent transport process. Hence the extra expenditure of energy on the internal pH maintenance causes growth inhibition and the growth may stop when the pH gradient collapses due to scarcity of catabolic energy meant for preventing the influx of undissociated acid in the cell (Bigelis et al., 1994). Effects of neutralizers such as NaOH, $CaCO_3$ and NH_4OH and their lactate salts on biomass growth and lactic acid production have been reported by (Hongo et al., 1986) Thus suitable neutralizing agents for pH control in fermentation broth during lactic acid production becomes necessary for conversion of undissociated lactic acid to their corresponding lactate salts.

3. Materials and Methods

The chemicals used in the present studies were of S. d. Fine, Qualigen and Merck make. Pure cultures of lactic acid bacteria, *Lactobacillus delbrueckii* NCIM 2025 and *L.pentosus* NCIM 2912 obtained from National Chemical Laboratory, Pune, India, were stored in MRS agar slants and subcultured monthly as directed. For carrying out the experiments on glucose based media, the above mentioned pure strains of *Lactobacilli* and their co-culture were precultured in MRS medium at 30°C and 150 rpm for 9 hours. The co-culture of *Lactobacilli* was prepared by using equal portions of inoculum dose from the two pure strains in the MRS media at 30°C and 150 rpm for 9 hours. Composition of one litre MRS medium was: 10g proteose peptone, 5g yeast extract, 10g beef extract, 20g dextrose, 1g tween 80, 2g ammonium citrate, 5g sodium acetate, 0.1g $MgSO_4.7H_2O$, 0.05g $MnSO_4$, 2g K_2HPO_4. One litre of glucose based synthetic media consists of : 60g glucose, 1.5g yeast extract, 0.1g sodium acetate, 0.05g KH_2PO_4, 0.05g K_2HPO_4, 0.02g $MgSO_4$, 0.003g $MnSO_4$, 0.003 $FeSO_4$. Two sets of fermentation experiments were carried out taking both the *Lactobacillus* pure cultures and the co-culture at 37°C, 180 rpm, 1.55 g/l (cell dry weight) inoculum with pH maintained at 5.60 at every 12 h with either 2% NaOH or 2% $CaCO_3$ as neutralizing agent. Initial pH was kept at 6.5 for each experiment. Biomass (cell dry weight) during fermentation in synthetic media was determined at every 12 h interval. The fermentation broth was centrifuged at 8000 rpm for 10 minutes to separate the cells from broth, leaving the supernatant. The precipitated cells were washed with 0.85% NaCl (to remove any remaining substrate) and were dried in preweighed

microporous papers at 70°C till attainment of constant weight. The pH values of fermentation broths due to acid production were recorded at every 12 h interval with the help of a digital pH meter.

4. Results and Discussions

The results of the batch experiments on glucose based synthetic media treated with NaOH or $CaCO_3$ as neutralizers have been provided in Table-1 and 2. For the first set of experiments using NaOH as neutralizer on glucose based synthetic production media (initial pH 6.5) with *Lactobacillus sp.* pure cultures and co-culture Fig.1 and Table 1 indicate that highest biomass (cell dry weight) production exists with co-culture. The co-culture has shown a higher pH drop (hence higher acid formation) with respect *Lactobacillus* pure cultures through the course of fermentation. From Fig. 1 and Table 1 it is also evidenced that during 24 to 60 h period for pure cultures and 24 to 48 h duration for co-culture, there is gradual increase of biomass (cell dry weight) every time accompanied by sharp pH drops from pH 5.6 indicating higher acid production which is probably because the cells were in a physiological state that favours more towards acid production than the biomass growth. This duration also includes the stationary phase of the batch curve. It is observed from Fig. 1 that, the stationary phase for the co-culture occurs later than the pure culture strains as the cells of the co-culture were actively engaged both in growth and acid synthesis. The co-culture had longer log phase, which can serve as significant basis for its use in industrial fermentation. The higher pH drops effected by pure strains and co-culture every 12 h as mentioned in Table 1, can be attributed to the beneficial effects of periodic make up of pH up to 5.6 every 12 h with NaOH as neutralizer to minimize

Table 1: Biomass growth and pH drop effected by *Lactobacillus sp.* pure strains and their co-culture with NaOH as neutralizer.

Time (h)	*L. delbrueckii*		*L. pentosus*		Co-culture	
	Biomass, g/l	pH	Biomass, g/l	pH	Biomass, g/l	pH
0	1.55	6.50	1.55	6.50	1.55	6.50
1.25	1.56	6.35	1.60	6.30	1.95	6.26
12	4.88	5.20	9.69	5.50	20.60	5.28
24	6.69	4.80	11.51	5.30	22.06	4.68
36	6.68	4.79	11.40	4.55	23.95	4.60
48	6.16	4.70	10.44	4.64	23.90	4.50
60	5.08	4.76	9.69	4.85	18.23	4.68
72	4.88	4.95	8.95	4.88	15.12	4.78
84	4.82	5.12	8.10	5.25	14.10	4.96
96	3.51	5.09	7.27	5.44	13.50	5.08
108	1.56	4.98	3.92	5.40	12.07	4.93

product inhibition in acid synthesis. In 84 h to 108 h duration the pH drop effected by *L.delbrueckii* is higher than the *L. pentosus* possibly due to higher temperature adaptability of *L. delbrueckii* (37 to 42 °C).

Table 2 : Biomass growth and pH drop effected by *Lactobacillus sp.* pure strains and their co-culture with $CaCO_3$ as neutralizer.

Time, h	*L. delbrueckii*		*L. pentosus*		Co-culture	
	Biomass, g/l	pH	Biomass, g/l	pH	Biomass, g/l	pH
0	1.55	6.50	1.55	6.50	1.55	6.50
1.25	1.56	6.42	1.60	6.46	1.98	6.40
12	4.20	5.60	4.64	5.44	11.52	4.88
24	7.25	5.28	5.06	5.20	12.36	5.34
36	8.95	5.16	12.61	4.88	15.75	5.14
48	9.09	4.80	12.89	4.70	16.08	5.38
60	5.36	4.89	10.01	5.22	10.04	5.42
72	4.13	5.30	8.51	5.18	8.82	5.40
84	3.64	5.27	7.55	5.10	7.25	5.43
96	3.12	5.23	7.10	5.06	6.34	5.47
108	3.05	5.14	4.89	4.71	5.46	5.48

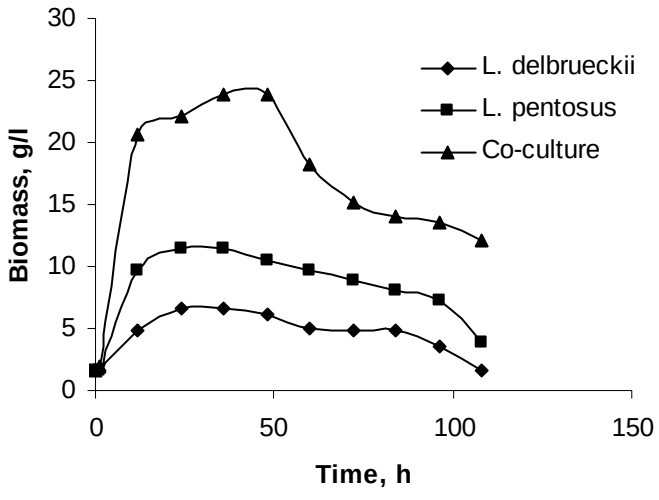

Figure 1: Variation in biomass growth with progress of fermentation in synthetic production medium with NaOH as neutralizer.

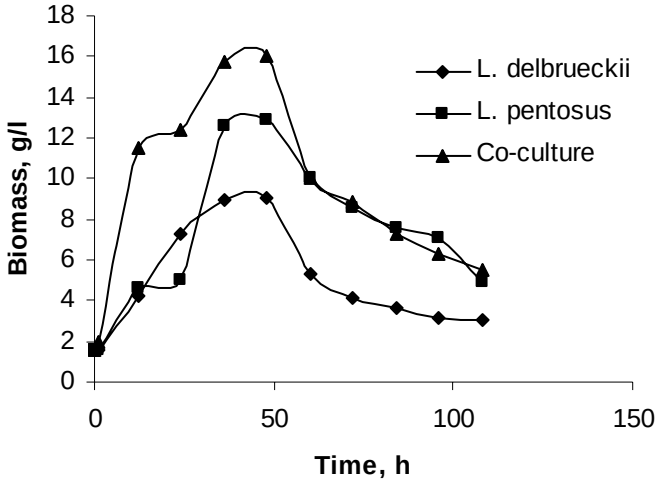

Figure 2: Variation in biomass growth with progress of fermentation in synthetic production medium with $CaCO_3$ as neutralizer.

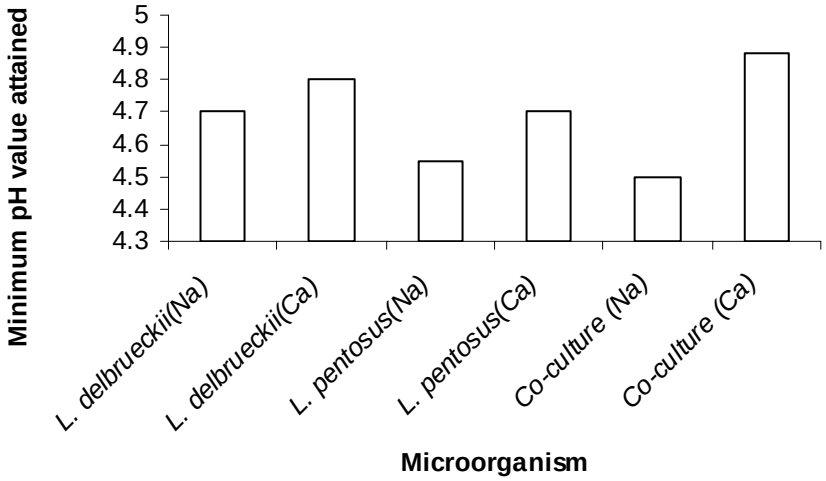

Figure 3: Minimum pH attained by Lactobacillus pure cultures and their co-culture in synthetic production medium with NaOH or $CaCO_3$ as neutralizer.

It is observed from Table 1 and Fig. 1 that the co-culture bears superiority over the *Lactobacillus* pure strains in synthetic production media with NaOH, because it exhibits advantageous traits such as, (i) higher biomass concentration (indicating

higher growth rate) at the end of log phase, which is accompanied with lowest pH drop value (hence higher acid synthesis), (ii) has longer log phase than the pure strains, with more biomass and higher pH drop values and (iii) better acid tolerance, as it produces high biomass even at low pH value in NaOH containing fermentation broth. In the first set of batch experiments with *L. delbrueckii, L. pentosus* and co-culture the pH drop in synthetic production medium with NaOH treatment, increased with increasing biomass formation in the log phase while the pH drop decreases with decrease in biomass concentration from stationary phase onwards till end of fermentation, which indicates reduction in acid production.

In the second set of batch experiments with $CaCO_3$ as neutralizer it can be observed from Table 2 and Fig. 2 that there is over all increase in biomass growth of pure *Lactobacillus* cultures as compared to those attained in the batch experiments with NaOH as neutralizer. Above observation can be due to the fact that calcium ions take part in cell division in some *Lactobacillus* species, as also reported earlier (Kojinin et al., 1970). However, the fall in pH values (hence acid formation) is lesser with $CaCO_3$ as compared to that in case of NaOH is evidenced from Fig. 3. Huang et al. (2004), reported a similar observation regarding fall in lactic acid yield above 1% $CaCO_3$ treatment of media in case of *Rhizopos oryzae* and *R. arrhizus*. Wantanabe et al. (1972) reported the roll of calcium ions in phage infection of *Lactobacillus sp*. cells, which may also be a possible reason for lower acid production with $CaCO_3$ treatment. It is observed from Table 2 that *Lactobacillus* pure cultures effect a higher fall in pH value as compared to the co-culture in presence of $CaCO_3$. The above observations show that (i) In presence of $CaCO_3$ the *Lactobacillus* cells attain a physiological state where the biomass growth is encouraged more than the acid formation. (ii) The co-culture showed lesser compatibility with $CaCO_3$ treatment in terms of acid production as compared to its performance in NaOH treated synthetic media. It is evident from the pH drop values of *L. delbrueckii* and *L. pentosus* given in Table 1 and Table 2, that NaOH treatment proves better for *L. delbrueckii*. Although *L. pentosus* attained the least pH value of 4.55 with NaOH treatment but it performs better in terms of pH drop (acid formation) in the latter stages (84 - 108 h) of fermentation in case of $CaCO_3$ treatment. Thus a higher acid synthesis activity was achieved for a longer duration by *L. pentosus* during fermentation in case of $CaCO_3$ treatment.

5. Conclusion

The results of the experiments with NaOH showed that the co-culture produces more acid than the pure strains of *Lactobacillus,* whereas in case of $CaCO_3$ treatment acid production was higher with pure strains. With NaOH as neutralizer lower pH values were evidenced in case of co-culture for a longer period of time (24 - 108 h). The lower values of biomass and acid production, in the second set of experiments suggest that $CaCO_3$ has inhibitory effect on the performance of co-culture.

6. References

Adsul M.G., A.J.Varma and D.V. Gokhale, 2007, Lactic acid production from waste sugarcane bagasse derived cellulose, Green Chemistry 9, 58.

Altaf,M., B.J. Naveena and G. Reddy, 2007, Use of inexpensive nitrogen sources and starch for L(+) lactic acid production in anaerobic submerged fermentation, Bioresource Technology 98, 498.

Bigelis, R., and S.P. Tsai, Microorganisms for organic acid production, Food biotechnology: Micro organisms, Y.H. Hui ed., 1994, Wiley- IEEE.

Calabia, B.P. and Y.Tokiwa, 2007, Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugarbeet juice by *Lactobacillus delbrueckii*, Biotechnology Letters 29, 1329.

Hongo, M.,Y.Nomura and M.Iwah, 1986, Novel method of lactic acid production by electrodialysis fermentation, Applied and Environmental Microbiology 52, 316.

Huang, L.P., B. Jin, P. Lant and J. Zhou, 2004, Simultaneous saccharification and fermentation of potato starch waste water to lactic acid by *Rhizopus oryzae* and *Rhizopus arrhizus*, Biochemical Engineering Journal 23, 269.

Jennings, D.H., 1995, Primary active transport, The physiology of fungal nutrition, Cambridge University Press, Cambridge.

Kojinin, M., S. Suda, S. Hotta, K. Hamada and A. Sunganuma, 1970, Necessity of calcium ion for cell division in *Lactobacillus bifidus*, Journal of Bacteriology 104, (2), 1010.

Mirdamadi, S.,H. Sadeghi, N. Sharafi, M. Fallahpour,F.A. Mohseni and M.R. Bakhtiari, 2002, Comparison of lactic acid produced by fungal and bacterial strains, Iranian Biomedical Journal 6, (2&3), 72.

Narayanan, N., P.K. Roychoudhury and A. Srivastava, 2004a, L(+) Lactic acid fermentation and its product polymerization, Electronic Journal of Biotechnology 17, (2), 4.

Narayanan, N., P.K. Roychoudhury and A. Srivastava, 2004b, Isolation of adh mutant of *Lactobacillus rhamnosus* for the production L (+) Lactic acid, Electronic Journal of Biotechnology 17, (1), 2.

Pieterse,B., R.J. Leer, F.H.J. Shuren, and M.J. Vanderwerf, 2005, Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling, Microbiology 151, 3881.

Pressor, K.A., D.A. Ratkowsky and T. Ross, 1997, Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration, Applied and Environmental Microbiology 63, (6), 2357.

Ross,P.R., S. Morgan and C.Hill, 2002, Preservation and fermentation: Past, present and future, International Journal of Food Microbiology 79, (1-2), 3.

Sherman, A.S., Y.X. Li and J.E. Keizer, 2006, Whole cell models, Computational Cell Biology, Springer, Delhi (First Indian Reprint).

Todorov, S.D., M. Botes, S.T. Danova and L.M.T. Dicks, 2007, Probiotic properties of *Lactobacillus lactis ssp.lactis* HV219 isolated from human vaginal secretions, Journal of Applied Microbiology 103, 629.

Wantanabe ,K. and S. Taksue, 1972, The requirement of calcium in infection with *Lactobacillus* phage, Journal of General Virology 17, 19.