

## Screening of Bacteria for Lactic Acid Production from Whey Water

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### ABSTRACT

*Lactobacilli* have the property of converting lactose and other sugars to lactic acid through fermentation. So whey water, the greenish translucent liquid rich in lactose, vitamins, proteins and mineral salts, obtained as a by-product after the precipitation of cheese can be used as a substrate for *Lactobacilli* for lactic acid production which otherwise is a serious environmental pollutant when disposed without pre-treatment. 16 isolates of Lactic acid producing bacteria isolated from various biological sources were inoculated in whey water (1% inoculum) and kept at 37°C in the shaker at a speed of 150 revolutions per minute for 36 h. Lactic acid production was estimated after 36 h and the strains 4a, 12a and 15b showed lactic acid production of which 12a produced the highest concentration. The amount of Lactic acid produced by 12a was 0.62 g L<sup>-1</sup> under unadjusted condition which is comparable to previously reported strains in enriched medium. So the lactic acid production by strain 12a was further investigated to find the effect of pH and temperature on the production efficiency. Lactic acid production was also checked in Luria-Bertani broth and whey water was found to be the medium of choice for prolonged lactic acid production.

**Keywords:** *Lactobacilli*, Lactic Acid, Whey Water, Biological Oxygen Demand

### 1. INTRODUCTION

*Lactobacilli* are gram positive (Abedon, 1998), non spore forming cocci, coccobacilli or rods. Since these organisms have the property of producing lactic acid from lactose and other carbon sources through fermentation they are classified as Lactic Acid Bacteria (LAB). All the members of this group can grow anaerobically and they can also tolerate oxygen due to the presence of superoxide dismutase which detoxifies the free radicals and hence the name aero tolerant anaerobes. LABS obtain energy from the metabolism of sugars. So they are present only in environments containing sugar like in the oral cavity, the intestinal tract and the vagina (Dicks *et al.*, 2000). They produce antimicrobial peptides called Bacteriocins which

help this group of organisms to kill the harmful microorganisms around the environment. Lactic acid bacteria have anti-inflammatory and anti-cancer activity (Chen *et al.*, 2009). Few of the *Lactobacilli* are pathogenic to animals. In humans *Streptococcus pneumoniae* causes lobar pneumonia, otitis media and meningitis (Aguirre and Collins, 1993); some viridans and nonhemolytic oral streptococci play a role in dental caries (Tasli *et al.*, 2006).

LAB produce organic (acetic and lactic) acid which inhibits the growth of many bacteria especially pathogenic gram negative bacteria. The antimicrobial activity occurs through the diffusion of lactic molecules into microbial cells until equilibrium is reached, in accordance with the pH gradient, causing membrane disruption, inhibition of essential metabolic reactions,

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stress on intracellular pH homeostasis and accumulation of toxic anions and ultimately death of microbial cells (Brul and Coote, 1999). Besides the anti-microbial activity Lactic acid also has wider applications in food, cosmetic, pharmaceutical and chemical industries because of the increasing market demand for the naturally produced lactic acid (i.e., 130,000-150,000 tonnes/year) (Mirasol, 1999). The fact that 10% of lactic acid produced is by synthetic means from Lactonitrile, it would be better to recycle the industrial wastes like whey water which are rich in lactose for Lactic acid production which otherwise pollutes the environment.

Whey water, the greenish translucent liquid obtained after the precipitation of cheese is a very good source of lactose, proteins, vitamins and minerals which in turn is responsible for the foul smell generated during decomposition of it upon discharge (Mukhopadhyay *et al.*, 2003). Every year  $10^8$  tonnes of whey water is produced and for 1 Kg of cheese produced 9 litres of whey water is obtained as effluent (Zafar and Owais, 2006). When this whey water is disposed to the environment without pre-treatment, it pollutes the water bodies by increasing the biological oxygen demand in the range of 38,000 to 46,000 ppm as opposed to 200 ppm in case of sewage (Marwaha and Kennedy, 1988; Mawson, 1994). About 1, 50, 000 tonnes of cottage cheese and 2 million tonnes of whey (with about 1,30,000 tones of milk nutrients) are produced annually in India (Dernirel *et al.*, 2005). These were some of the facts that led to the formation of the Environmental protection Act-1986 by the Government of India making it obligatory to pre treat the dairy effluent (whey) before discharging into the environment.

This study concentrates on the isolation of Lactic acid Bacteria from various biological sources and screening the 16 strains for lactic acid production in both Whey water and Luria-Bertani (LB) broth and immobilising the strains with higher lactic acid production for the continuous generation of lactic acid.

## 2. MATERIALS AND METHODS

### 2.1. Screening of Lactic Acid Bacteria

For screening, total numbers of LAB colonies were counted after overnight incubation of samples in LB agar plates containing 0.4%  $\text{CaCO}_3$  at 37°C. List of samples collected were as follows: (1) Milk, (2) Cheese, (3) Fruit Juice, (4) Sambar, (5) Chutni, (6) Sweet yoghurt, (7) Sour yoghurt, (8) Country Liquor, (9) Idli Batter, (10) *Labeo rohita* (intestine), (11) *Raiamas bola*. (intestine), (12) *Catla catla* (intestine), (13) *Penaesus indicus* (Flesh), (14) Probiotic (prowel), (15) Probiotic (Laviest), (16) Probiotic (Binifit), (17) Packet Milk (pasteurized), (18)

Pastry, (19) Buttermilk, (20) Mango pickle, (21) Chilli pickle, (22) Pickle filler, (23) *Solanum tuberosum*, (24) *Solanum lycopersicum*, (25) *Malus domestica*. (Juice), (26) *Musa acuminata* (rotten), (27) Grapes (rotten), (28) *Mangifera indica* (rotten), (29) *Solanum melongena* (rotten), (30) *Allium cepa*, (31) *Oreochromis niloticus niloticus* (intestine), (32) *Heteropneustes fossilis*. (intestine), (33) *Sus scrofa domesticus* (flesh), (34) *Sus scrofa domesticus* (intestine), (35) *Puntius* sp. (intestine), (36) *Gallus domesticus*(flesh), (37) *Gallus domesticus* (intestine), (38) *Capra hircus* (meat), (39) *Capra hircus* (intestine), (40) Long grass, (41) Grass (shoot), (42) Bitter gourd grass, (43) Curry leaves, (44) Tomato leaves.

Small portion of each samples were mixed with sterile Phosphate Buffer Saline (PBS). The leaf samples were sonicated in 5 mL PBS for 14 min at maximum frequency (not to lyse the cells). The samples were serially diluted to  $1 \times 10^{-8}$  dilution and 50  $\mu\text{L}$  of each of the samples were spread in LB agar plates containing 0.4%  $\text{CaCO}_3$ . The Petri plates were kept in inverted position for overnight incubation at 37°C. The Lactic acid bacteria were demarcated by having a clearing zone.

### 2.2. Starter Culture

16 *Lactobacilli* strains from glycerol stock were inoculated in 2 mL Luria-Bertani medium and allowed to grow for 24 h at 37°C in a shaker which runs at a speed of 150 revolutions per minute.

### 2.3. Preparation of Whey Medium

Whey medium was prepared by boiling the milk and then precipitating the casein in the milk by adding citric acid (for 250 mL of milk, lime juice obtained from one lemon was used). The whey water obtained by this method was purified by centrifuging it at 16000 g for 10 min followed by filtering through a 0.45  $\mu$  filter. 2 mL of the pure whey water was transferred into sterile tubes inside laminar flow hood. The pH of the whey media was between 4.5-5.0 and no nutrient supplements were added. The media was now ready for inoculation of isolated strains.

### 2.4. Preparation of LB Medium

LB medium was prepared by adding 1% Tryptone, 0.5% yeast extract and 0.5% sodium chloride in water with pH of 7.5 and autoclaved at 121°C for 15 min.

### 2.5. Inoculation of Culture

Each tube containing whey medium and LB medium was inoculated with the 16 strains (1% inoculum) separately and allowed to grow for 36 h in the case of whey water and 24 h in the case of LB at 37°C in a shaker

which runs at a speed of 150 revolutions per minute. After respective period of incubation, the cultures were taken out and the Lactic acid test was performed.

## 2.6. Lactic Acid Test

Lactic acid was measured by modified Barker and Summerson (1941) method. Lactic acid was first oxidised with strong Sulphuric acid solution to acetaldehyde and then it was coupled with p-hydroxy diphenyl in the presence of cupric ions to yield a purple compound. The absorbance of purple compound was measured using spectrophotometer at 560 nm. In biological material the protein content must be first precipitated prior to measuring the Lactic acid concentration.

## 2.7. Preparation of Protein Free Supernatant

The sample was made protein free by treating the supernatant which was obtained after centrifugation (16000 g for 10 min) with 100  $\mu$ L of 100% Trichloroacetic acid for each 1 mL of sample. This was followed by incubation in ice for 30 min and centrifugation at 16000 g for 5 min for collection of protein free supernatant.

## 2.8. Treatment with Copper and Calcium

Treatment with copper sulphate and calcium hydroxide is done to remove any interfering material. 1 mL of the protein free supernatant obtained by the above method was diluted to 9 mL with distilled water and 1 mL of 20% copper sulphate was added to this followed by addition of 1gm of calcium hydroxide. The mixture was shaken vigorously. The above mixture was allowed to stand at room temperature for atleast half an hour with occasional shaking followed by centrifugation at 16000g for 10 min.

## 2.9. Colour Development

To 1 mL of supernatant obtained after centrifugation, 0.05 mL of 4% copper sulphate solution and 6 mL of concentrated sulphuric acid were added with continuous mixing. The mixture was boiled in 100°C water bath for 5 min followed by placing in cold water. After cooling 0.1 mL of alkaline solution of p-hydroxy diphenyl was added and the tubes were placed in 30°C water bath for 30 min. The tubes were again placed in 100°C water bath for 90 sec to dissolve the excess reagents. The above solution was then cooled and the absorbance was taken at 560 nm. The concentration of lactic acid produced was estimated by using known concentration of sodium lactate as standard.

## 2.10. Optimisation of pH and Temperature for the Screened Strain

### 2.10.1. Effect of pH

For optimising the pH the fermentation medium (whey water) was adjusted to five different pH (5.0, 5.5, 6.0, 6.5, 6.8) and kept in 37°C shaker which runs at a speed of 150 revolutions per minute and the Lactic acid production was checked for each pH after 12 h. The optimised pH was maintained for further work.

### 2.11. Effect of Temperature

Whey water with adjusted pH was maintained at five different temperatures (30, 35, 37, 40 and 45°C) by keeping them in the respective shakers running at a speed of 150 rpm and the lactic acid production was estimated after 12 h.

## 2.12. Continuous Generation of Lactic Acid in Whey Water and LB Broth

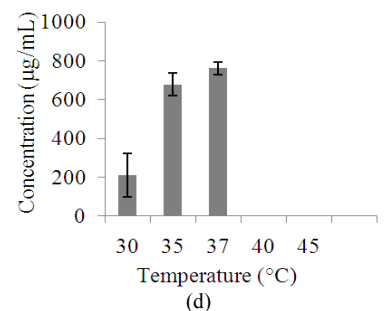
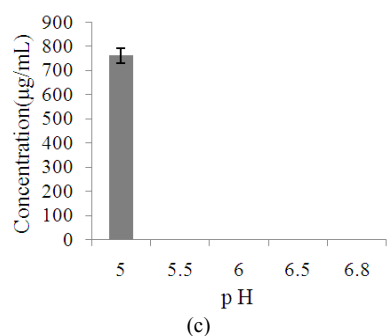
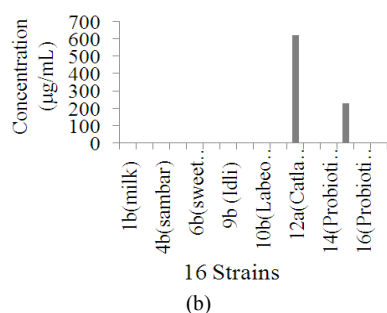
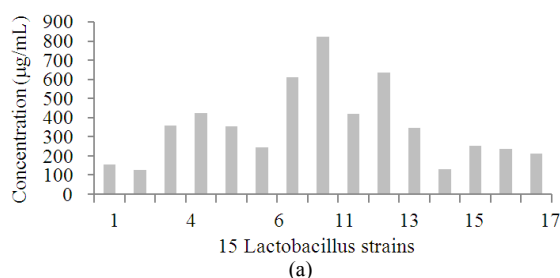
2.5 litres of whey water and LB medium containing 5% inoculum of isolate 12a were taken in respective bioreactors packed with corrugated sheets. The cultures were allowed to grow for 18 h inside the bioreactor before being drained and kept dry for immobilization for 6 h. After 6 h, fresh whey water and LB were added to the respective. Lactic acid production was checked every 24 h and the whole process were repeated till there was a decline in lactic acid production.

## 2.13. Strain Characterization

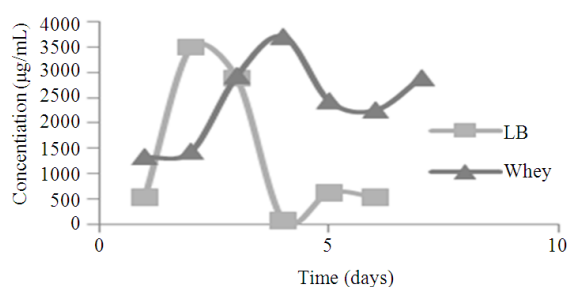
The isolate capable of producing lactic acid from whey water was characterized at length as per earlier reports (Nandy *et al.*, 2007). It was further characterized at the molecular level (16S rDNA) as per the method reported earlier (Chaudhuri and Thakur, 2006). The partial sequences obtained were subjected to Blast analysis and the novel sequences were submitted to GenBank.

## 3. RESULTS

Out of the forty four different samples that were tested for probable sources of lactic acid bacteria, not all were identified as potential sources of these bacteria. A comparison of the percentage of LAB obtained from the different sources can help us designate the sources according to the percentage of LAB obtained and hence the sources were classified as the most potential sources of LAB (from which >80% of LAB was isolated); potential sources of LAB (from which >60% of LAB was isolated); probable sources of LAB (from which >40% of LAB were isolated); least probable sources of LAB (from which <40% of LAB were isolated).



**Fig. 1.** Lactic acid production by bacterial isolates. (a) Lactic acid production in LB after 24 h of growth by the 15 LAB isolates namely 1b, 3b, 4b, 5b, 6b, 7b, 9b, 10a, 11b, 12a, 13b, 14, 15b, 16, 17 as per the source of isolation mentioned in the materials and method; (b) Screening of Lactic acid production by 16 selected strains (1b, 3b, 4b, 5b, 6b, 7b, 9b, 10a, 10b, 11b, 12a, 13b, 14, 15b, 16, 17) in whey water after 36 h of growth; (c) Effect of pH on lactic acid production by isolate 12a in whey water after 12 h of growth; (d) Effect of Temperature on lactic acid production by isolate 12a in whey water after 12 h of growth



**Fig. 2.** Comparison of lactic acid production by isolate 12a in Luria Bertani and whey water

In LB all the *Lactobacillus* strains had produced lactic acid of which isolate 10b had produced  $0.823 \text{ g L}^{-1}$  and isolate 12a had produced  $0.634 \text{ g L}^{-1}$  (**Fig. 1a**). Among the 16 strains checked 3 strains isolated from sambar (4b), *Catla catla*-intestine (12a), Probiotic-Lavies (15b) have produced Lactic acid after 36 h of which the production was too low in the sambar strain (4b). The concentration of Lactic acid produced by *Catla-catla* strain (12a) and Probiotic-Lavies strain (15b) were  $6.9 \text{ mM}$  ( $0.62 \text{ g L}^{-1}$ ) and  $2.5 \text{ mM}$  ( $0.23 \text{ g L}^{-1}$ ). Since 12a had produced higher amount compared to 15b (**Fig. 1b**), it had been screened for optimisation and continuous generation of lactic acid.

Since isolate 12a had produced lactic acid in both LB and Whey water, it was screened for optimization of lactic acid production in whey water and also for the continuous generation of lactic acid both in LB and Whey water in a packed bed bioreactor.

Isolate 12a produced more amount of lactic acid when the whey water pH was adjusted to 5.0 (**Fig. 1c**) and it was optimised for further work. The concentration of lactic acid produced was  $0.761 \text{ g L}^{-1}$ . Whey water culture maintained at  $37^\circ\text{C}$  (**Fig. 1d**) had produced higher amount of lactic acid compared to the other temperatures and the amount of lactic acid produced was  $0.761 \text{ g mL}^{-1}$ .

### 3.1. Immobilisation for Continuous Generation of Lactic Acid

On first day whey water ( $1.333 \text{ g L}^{-1}$ ) produced higher concentration of lactic acid compared to Luria Bertani broth ( $0.540 \text{ g L}^{-1}$ ) but on the second day production was higher in LB ( $3.495 \text{ g L}^{-1}$ ) compared to whey water ( $1.494 \text{ g L}^{-1}$ ). LB medium produced higher concentration of lactic acid within a short duration but for prolonged production of lactic acid, whey water would be a better carbon source for LAB isolate 12a (**Fig. 2**).



### 3.2. Strain Characterization

This strain (12a) was oxidase positive as well as gram positive cocci (at 100X magnification of Axiostar Plus, Zeiss). But at the molecular level it was closest to *Bacillus* sp (*Bacillus* sp. strain *SRCKk.01*) with the following taxonomic identity: Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus. The GenBank accession number of its partial 16S rDNA sequence is GQ979935. It lacks capsule, endospore, DNase, lipase, protease, catalase and lecithinase formation. Of the antibiotics tested [Ampicillin (A, 10 µg), Cephadroxil (Cq, 30 µg), Chloramphenicol (C, 30 µg), Cloxacillin (Cx, 10 µg), Cephotaxime (Ce, 30 µg), Ceftazidime (Ca, 30 µg), Ciprofloxacin (Cf, 5 µg), Doxycycline Hydrochloride (Do, 30 µg), Gentamicin (G, 10 µg), Metronidazole (Mt, 4 µg), Neomycin (N, 30 µg), Norfloxacin (Nx, 10 µg), Polymyxin B (Pb, 100 units), Rifampicin (R, 15 µg), Roxithromycin (Ro, 30 µg), Tetracycline (T, 30 µg), Trimethoprin (Tr, 30 µg), Vancomycin (Va, 30 µg)], the strain was sensitive to Chloramphenicol, Ampicillin, Roxithromycin and Cloxacillin. The minimum inhibitory concentration for metals like copper, lead, nickel and chromium in solution was 6mM, 7mM, 2mM and 1mM respectively. It could grow in a temperature range of 20 to 37°C with optimum growth at 20°C. The optimum pH for growth of the cell was 6 to 7.5. It could grow equally well in presence and absence of light. It could utilize the following carbohydrate sources: maltose, fructose, dextrose, galactose, sucrose, mannose, ribose, esculin, D-arabinose, citrate and malonate.

### 4. DISCUSSION

We observed that the various milk products and milk itself has the maximum content of lactic acid bacteria. Though pastry and buttermilk are also milk products but LAB could not be found may be because these are processed food products. Out of the different animals and fishes tested only intestine and flesh of *Gallus domesticus*, intestine of *Capra hircus* and intestine of *Puntius* sp. can be categorized as the sources from which 100% of LAB was isolated. Pickles are considered as good sources of LAB but in our experiment we could not isolate any LAB from them. This may be because of the high salt content used for the preservation of pickles which in turn makes the survival of LAB unfavourable. Fruits were also shown to be poor sources of LAB. Out of the grasses and vegetables that we chose, only the long grass variety was observed to be a potential source of LAB.

*Catla catla* strain (12a) had produced 0.62 g L<sup>-1</sup> of lactic acid after 36 h even at a pH of 4.63 and without any nutrient supplements. After adjusting the pH and temperature, the strain produced 0.761 g L<sup>-1</sup> of lactic acid after 12 h. But *Lactobacillus caesi* had produced 33.73 g L<sup>-1</sup> of lactic acid after an incubation period of 36 h with added nutrient supplements like yeast extract, Magnesium sulphate, calcium carbonate under optimised process parameters.

Lactic acid production was observed only in pH 5.0 and this is due to the ability of this particular strain 12a to survive under acidic conditions. A pH range of 6.0-6.5 has been optimised for *L. caesi*. However, in the case of *L. helveticus* the optimised pH was 5.5. Lactic acid production was observed at 30°C, 35°C, 37°C but the production was high at 37°C and this is the optimised temperature for this isolate 12a. The optimal temperature for *Lactobacillus* varies between 20-45°C.

In 2.5 L bioreactor both whey water and LB has started producing lactic acid after 24 h. In whey water the production was higher on the fourth day (3.69 g L<sup>-1</sup>) and then it shows a zig-zag pattern and this could be due to the shedding of Bio-film. In LB the highest production was observed on the second day (3.495 g L<sup>-1</sup>) and then it started declining and this could be due to accumulation of toxins and by products. Thus whey water can be utilised for the continuous generation of lactic acid.

### 5. CONCLUSION

The potential of using this isolate for bioremediation of dairy waste (whey water) could be explored and exploited in future. This would ensure conversion of waste product into a by product with immense commercial application.

### 6. ACKNOWLEDGEMENT

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