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ISOLATION AND CHARACTERISATION OF LACTIC ACID PRODUCTION AND ITS OPTIMIZATION UNDER SUBMERGED FERMENTANTION BY Lactobacillus sp

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Abstract

The aim of this study was to isolate Lactobacillus from different curd samples. A samples were collected from the local area Ozar. From these, isolates were obtained by growing on de Man, Rogosa anSharpe (MRS) agar medium.Dahi (curd) is a fermented milk product, most commonly used by Indian population. Trials are in process to establish dahi as a source of health beneficial organisms (probiotics).Lactobacillus acidophilus not only improves the intestinal flora balance but also inhabits the growth of undesirable microorganisms in intestine, which is benefit to the health of humans and animals.Dahi (curd) is a fermented milk product, most commonly used by Indian population. Trials are in process to establish dahi as a source of health beneficial organisms (probiotics). In this study, LAB in dahi was isolated using the man Rogasa sharge (MRS) medium. The Isolates were then identified based on their morphology & biochemical properties. The tests conducted include: gram staining, biochemical tests. we isolated lactic acid bacteria from curd. The effect of different parameters such as PH of the medium, temperature. On the basis of physiological tests and sugar utilization pattern, all the three isolates were confirmed to the different species of Lactobacillus:Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus lactis. Among isolates, L. acidophilus was found to be prevalent in dahi Inoculum size. Incubation time and shaking speed were optimized to enhance the conversion of whey sugar into lactic acid. The optimum condition was found for fermentation with the process condition of PH 6.5. temp 37°c & Inoculum size 4%. (v/v) with an incubath of 120h & effective rotation speed of 150. The above mentioned optimizes process parameters can be used. In large scale product of lactic acid fermentation" in further investigation by using curd at a Substrate. Lactobacillus acidophilus not only improves the intestinal flora balance but also inhabits the growth of undesirable microorganisms in intestine, which is benefit to the health of humans and animals.

Key words: Lactic acid, Fermentation, Lactobacillus sp

Introduction

In recent years, with the awareness of the therapeutic effect on human health of consuming probiotic bacteria increasing, Lactobacillus acidophilus is a species of probiotic bacteria widely used as health foods and fermented milk. Dairy starter cultures[1].

Generally, the healthiness of food has been linked to a nutritionally rich diet recommended by specialists and the role of it in totality has been emphasized instead of emphasizing on Individual components.

Milk, very first food of mammals including humans, is surrounded with emotional & cultural importance in the society .Men have been habituated to think of milk as nature's most perfect food for them. Therefore, milk & dairy products have long been recognized as an important constituent of balanced diet for human beings as these products provide wide range of essential nutrients[2].

The microbial ecology in the gastrointestinal tract influences many functions our body (i.e,digest" absorpt" of nutrients, detoxificat"& ultimately the functioning of immune system). All these aspects make the gut a target organ for development of functional foods that can help in maintaining the relative balance of microorganism in the gastrointestinal tract. The establishment of microbial balance by shifting it towards a beneficial one with the help of specific dietary components (ie. Probiotics and prebiotics) has opened the gateway for the development of Functional foods ensuring more benefits to the host's health. Probiotics are defined as live minimum which transit the gastro-intestinal tract in doing so, benefit the health of the consumer. Probiotic micro-organisms are found in many food products, especially in the fermented foods. Therefore, the probiotic lactic acid bacteria can be isolated from the fermented milk. products like acidophilus milk, yoghurt lactic cultures. Apart from the starter lactic cultures, dahi can also have some probiotics like Lactobacillus acidophilus Lactobacillus bulgaricus etc. Therefore, dahi can be used as a source for isolation of probiotic bacteria. The also a product of immense importance for human consumption, MRS broth for 24h at 37°c. Before inoculation of sample the PH of MRS broth was adjusted to 6.5 to 2. The enriched samples were on the petri plates. containing Lactobacillus select" MRS agar with the help of calibrated inoculating loop & incubated aerobically at 37°C for 48h and observed for the growth of colonies. Dahi naturally fermented milk by characterization of Lactobacili isolates grown on MRS agar was done mainly with the help of the following tests microscopic examination Gram Staining, catalase test, growth at different temp, Biochemical test & results were noted as Positive or Negative[3].

MATERIALS AND METHODS

Curd sample were collected by Ozar dist, Nashik, India were used for isolation Lactobacillus spp by serial dilution" method.

Method-

1g of curd sample was suspended in 9ml of normal saline & was vortexed for proper mixing & 0.1ml was spread on MRS agar plates & Incubated at 37°c for 48 hours & observed for the growth of Colonies. Pure

culture of Lactobacillus spp was subcultured In slants & petriplates Containing MRS agar broth & maintained in refrigerator at 4°c[1].

Colony Characteristics, Biochemical test of Lactobacillus spp.

Gram's staning Requirements:-

1) Young culture of microorganism
2) Crystal violets
3) Gram's iodine
4) Alcohol
5) Saffranin 6)D/W

METHOD:

1. Prepare a heat fixed omear of the culture.
2. Covers the smear with crystal stain for 1 min.
3. Add Gram's jodine to wash off crystal who let Stain & cover it with iodine till the ame turns coffee brown in colour (approve 1min)
4. Rinse the slide in running water.
5. Add decolorizing solution drop wise at upper end of slides held in inclined position till the violet color fails to from the smear for normal smear 10-15 sec. are enough.
6. Rinse the smear with water & observe in microscope.

Biochemical Test:-

1) Methyl Red(M-R) Test- REQUIREMENTS:

Methyl red indicator and Test culture

Procedure:

1) Inoculate MRVP broth with a pure culture of the organism.
2) Incubate at 35-37°C for 48hr
3) Add 5 or 6 drops of methyl red reagent
4) for the development of red colour
5) colour [if red colour forms, test is positive & if not test is negative[2].

Voges- Proskauer (V-P)-

REQUIREMENTS: KOH solution, Alpha-naphthanol 5%. Test culture, Barritt's reagent

Procedure:

1) Inoculate the pure culture & incubate at 37°C for 24-48 hr
2) After Incubat" add 6 drops of 5% Alpha-naphthanol & mix well to aerate.
3) Add 2 drops of 40%. potassium hydroxide & mix well to aerate.
4) Observed for a pink-red colour at the surface within 30 min, shake the tube vigorously during the 30min period[4].

CITRATE UTILIZATION TEST

REQUIREMENTS: Simmon'scitrateagar slant and Test culture

PROCEDURE:

1) Streakheavilyonthesurfaceofagarslantandincubatetheslantat37^{0}cfor24-48hr.
2) Record the color change of the slant after incubation
3) Observation[Green to blue = Positive, No change =Negative]

CATALASETEST REQUIREMENTS: Microscopic glass slide, 3%H2O2 and Test culture.

PROCEDURE:

1) Placeoneortwodropsofhydrogenperoxidesolutiononaglassmicroscopicslide.
2) Withanicromewirelooppickupcellsfromtheofawellisolatedcolonyofthetest.
3) Observe for the production of the gas bubbles of effervescence.

INDOLPRODUCTIONTEST

REQUIREMENTS: 1%TryptonebrothandErlich'sorKovac'sreagent and Testculture

PROCEDURE:

1) Take a sterilized test tubes containing 4 ml of tryptophan broth.
2) Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.
3) Incubate the tube at 37°C for 24-28 hours.
4) Add 0.5 ml of Kovac's reagent to the broth culture.
5) Observe for the presence or absence of ring.

Production of Lactic acid using submerged fermentation:

Productionof Lactic acid using submerged fermentation:

Materials: Lactobacillus spp. Culture plates, MRS broth and Rotary shaker.

PROCEDURE:

Method:

1) 20mlofautoclaved MRS broth inoculated loopful culture.
2) Thenitwasincubatedatroomtemperaturefor48hoursindark.
3) After48hoursitwascentrifugedat6000rpmfor10minutes.
4) Supernatant was used for screening of Lactic acid.

Screening of lactic acid(submerged fermentation)

Materials: Bacterial culture, strong sulphuric acid solution acetaldehyde, p-hydroxydiphenyl Distilledwater

UV/ Visible spectrophotometer.

Method:

1) At first, lactic acid was oxidized with strong sulphuric acid solution into acetaldehyde and then it was coupled with p-hydroxydiphenyl in the presence of cupric ions to yield a purple compound complex.

2) At 560 nm the absorbance of purple compound was measured using spectrophotometer[6].

Optimization of cultural parameters for enhanced production Lactic acid under submerged fermentation:

Parameter 1: Effect of Different pH on fermentation

Method:

The fermentation medium was adjusted to different pH (4.0, 5.0, 6.0, 6.5 and 8.0, 9.0) for optimizing and kept in shaker incubator at 37°C with rotating speed of 150 revolutions per minute. Lactic acid production was checked after 24 h. The optimized pH was maintained for further study.arameter 2: Effect of Temperature on fermentation

Method:

The optimum pH was maintained at five different temperatures (20, 25, 30, 37, 45 and 50°C) by keeping them with rotating of 150 revolutions per minute and the lactic acid production was estimated after 24 h.

Parameter 3: Effect of inoculum size on fermentation Method:

To study the influence of inoculum concentration on the lactic acid fermentation, different inoculum concentration (1-5%, v/v) were added sequentially to the fermentation medium and the lactic acid production was measured after 24 h.

Parameter 4: Effect of Incubation Period on fermentation

Method:

To find out the optimal time required for incubation to get maximal lactic acid production, the fermentative medium inoculated with bacterial culture was incubated for 24h, 48h, 72h, 96h, 120h, 144h and 168h respectively under the found above optimized conditions. At the end of each incubation period, lactic acid produced was estimated.

Parameter 5: Effect of rotation speed on fermentation

Method:

To study the influence of rotation speed on the lactic acid fermentation, different rpm at (50 to 200) were set sequentially to the fermentation process respectively under the found above optimized conditions and the lactic acid production was measured after 24 h[18].**Results and Discussion:**

Isolation and characterization

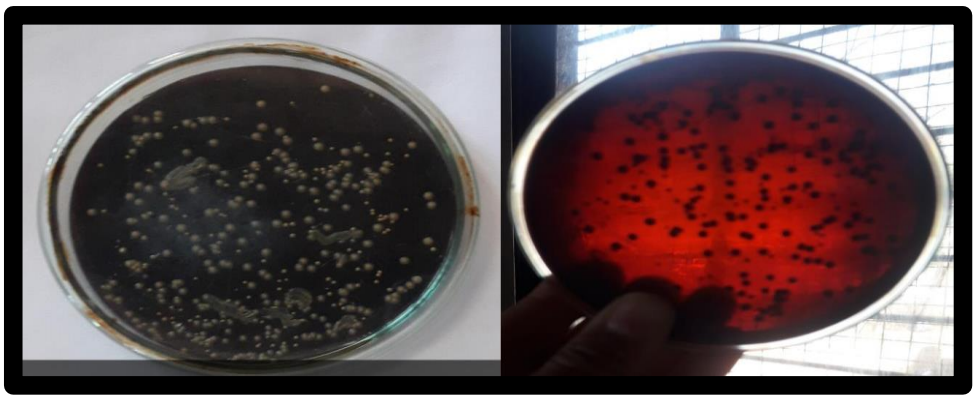


Fig.1: Isolation of lactobacillus bacteria by spread plate method, Lactobacillus spp. On MRS agar media

Colony characteristics:

Table 01: Colony characteristics

Parameters	**Observation**
Size	0.6 µm
Shape	rod
Colour	Cream white
Margin	Smooth
Elevation	Convex
Opacity	Opaque
Consistancy	Mucoid

Identification of lactic acid bacteria:

Biochemical test:

Gram staining:

Fig 02: The purple colour rod, Gram positive bacteria observed.

Table 02: Bergey‘s Manual of Determinative Bacteriology

Characteristics	Strain I	Strain II	*Lactobacillus acidophilus* Characteristics (Bergy's Manual)
Morphological Characterization			
Colour	Cream-white	Cream-white	Cream-white
Shape	circular	circular	Circular
Size	0.6µm	0.5µm	0.5-0.8µm
Motility	Non-motile	Non-motile	Non-motile
Gram reaction	Positive	Positive	Positive
Shape	rod	Rod	Rod
Physiological characterization			
Growth at different temperature			
15-20°C	-	-	-
30°C	+	+	+
35°C	+	+	+
37°C	+	+	+
Oxygen requirement			
Aerobic	+	+	+
Anaerobic	-	-	-
Microaerophilic	+	+	+
Biochemical characterization			
Indole	-	-	-
Methyl red	-	-	-
Voges proskauer	-	-	-
Citrate utilization	-	-	-
Urease	-	-	-
Triple sugar iron	-	-	-
Nitrate reduction	-	-	-
Catalase	-	-	-
Oxidase	-	-	-

Biochemical tests:-

Table 03: Biochemical tests

Sr. No	Test	Observation
1	Indol Test	Negative
2	Methylred Test	Negative
3	Vogesproskauer	Negative
4	Citrate Test	Negative
5	Catalase Test	Negative

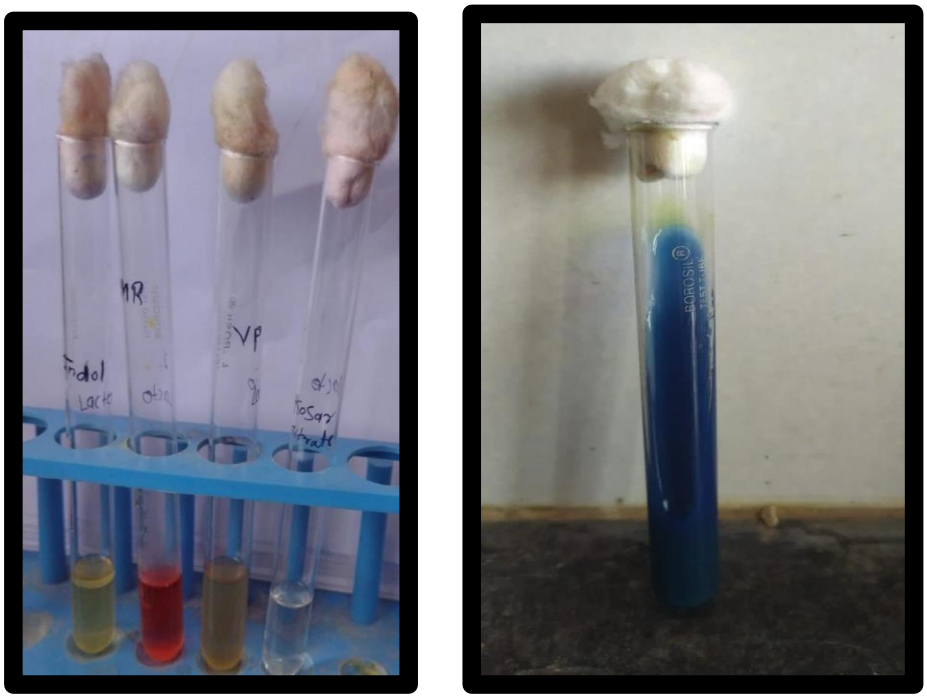


Figure 03: Biochemical test

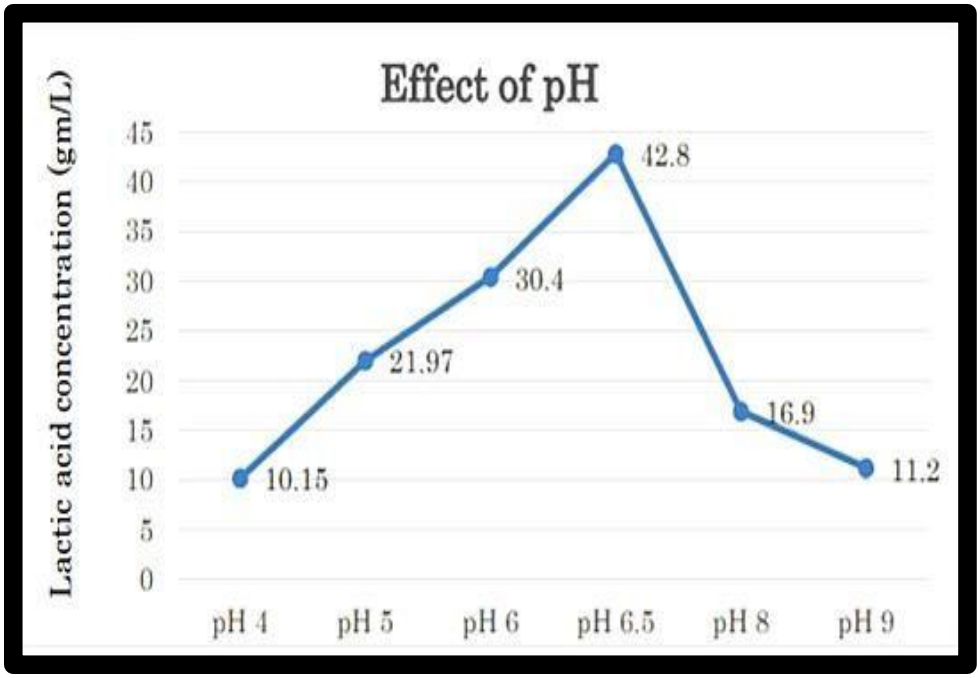
PARAMETERS:

Production of lactic acid µg/ml

Effect of PH:

Lactobacillus spp

The effect of pH on lactic acid production was estimated by using fermentation medium having a pH range of 4.0 -9.0 (Figure 1). The maximum lactic acid production (42.8 gm/L) was obtained at pH 6.5 on 24h of incubation. From pH 4.0 to 6.5 drastically increase the fermentative product, whereas after optimum pH 6.5, the lactic acid production sharply decreased that by using L. casei strain a pH range of 6.0-6.5 has been optimal for lactic acid production, which is supported to our obtained result reported that almost 95 % lactose conversion (w/v) corresponding to 33.48 gm/L lactic acid production was suitable at pH 6.5 All the above



findings, concluding that a pH 6.5 would be the optimal for maximum lactic acid production.

Figure 04: Effect of pH on lactic acid production

Effect of temperature:

To find the optimum temperature for lactic acid production, after adding whey into medium, inoculation was incubated at a temperature range of 25-50°C(Figure 2). The lactic acid production increased sharply with increase in the temperature from 25°C up to 37°C; and maximum production was found at 37°C(43.6 mg/L)however, an decrease in at 45°C (34.2 gm/L) and much lower of lactic acid production was found at 50°C (20.2gm/L). The temperature is also one of the important factors, which influences the activity of metabolic cell enzymes and every enzyme are most active at optimum temperature. In optimum temperature enzymatic reaction shows maximum reaction velocity. However, below and above the optimal temperature, reaction rate is slow down, which may effect on the cellular metabolism process.The optimal temperature for growth of lactic acid bacteria varies between the 20 to 45°C and obviously it varies on species to species used 37°C temperature for lactic acid production using L. casei reported maximum lactic acid production of 33.72 gm/L at 37°C . From the above observations, it is cleared that a temperature range of 37-40°C was considered optimal for lactose conversion to lactic acid using bacterial cells.

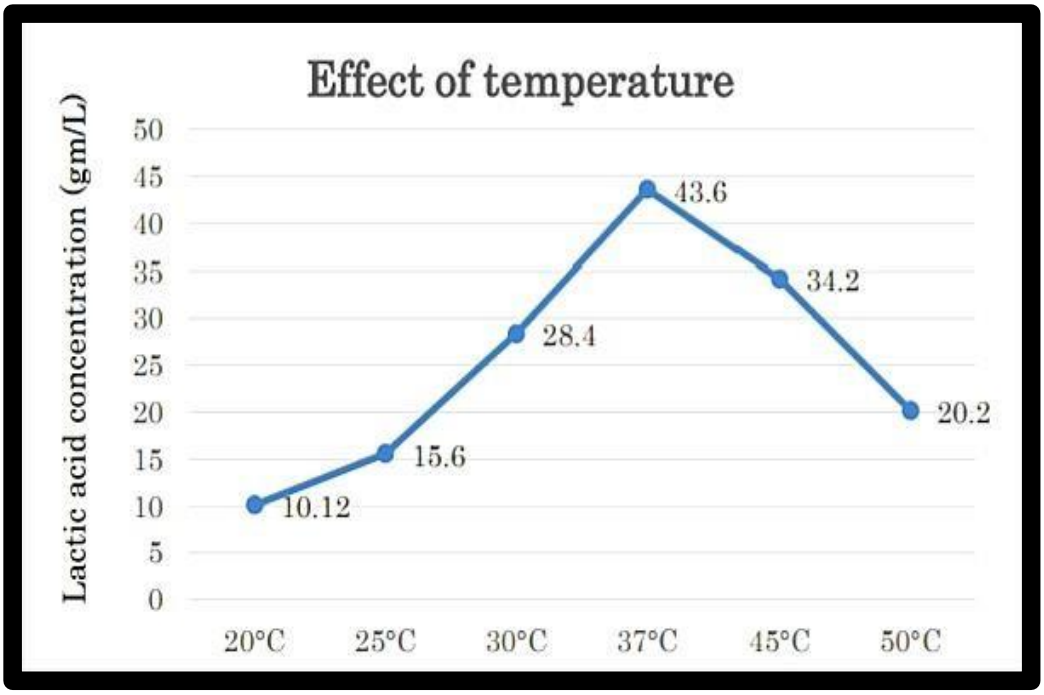
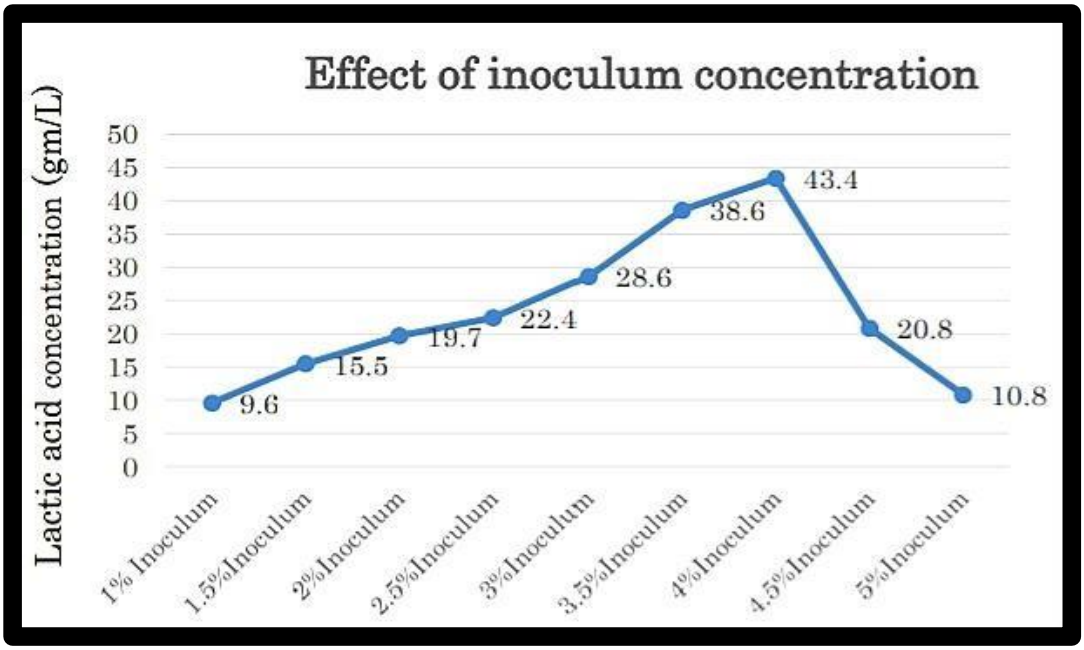


Figure 05: Effect of temperature on lactic acid production

Effect of Inoculum Size

To find out the influence of inoculum concentration on the lactic acid production, different inoculum levels (1-5%, v/v) were added to the fermentation medium (Figure 3). The lactose utilization and lactic acid production increased sharply with the rise in inoculum concentration up to 4% (v/v), thereafter no improvement in both the functions was observed by increasing the concentration of inoculum. The maximum lactic acid production of 43.4gm/L was observed with the adding of 4% (v/v) inoculums and later on production was lower down though increasing the inoculums concentration. At low density of starter culture (1%, v/v), the lowest lactic acid production was observed (9.6gm/L). The use of 2% (v/v) inoculum for the lactic acid production has been reported 3%, v/v inoculum for lactic acid production confirmed maximum lactic acid production of 2.52 gm/L with 4% (v/v) inoculum of bacterial culture which is supporting to our obtained result . From the above observations, an inoculum of 4% (v/v) could be considered optimal for achieving maximum lactic acid production using 24 h old bacterial culture and 4% (v/v) inoculum



concentration was used in the subsequent studies.

Figure 6: Effect of inoculum size on lactic acid production

Effect of Incubation Period

To find out the optimal incubation time for the maximal lactose utilization and lactic acid production, the whey medium inoculated with bacterial culture was incubated for different time at 24, 48, 72, 96, 120, 144 and 168 hour under the above optimized conditions. The samples were taken out at specified time intervals and the results obtained are presented in Figure. 4. As evident from the results, an increase in lactose utilization and subsequent lactic acid production was found increased till 120 h and thereafter sharply decrease both the activity was reported. This is due to the growth of the culture entered to the stationary phase and as a consequence of slow down the metabolic activity [16-17,29-31]. A maximum lactic acid production of 41.7 gm/L was observed after 120 h of incubation. Therefore, an incubation time of 120 h was considered optimal for lactic acid production in our case. Many researcher reported that incubation period of 48 h has been generally used for lactic acid production using different lactobacilli cultures [18-19,32-35].Though the reduction in fermentation period is additionally advantageous to improve the economics of the process, according to our obtained result, still we used, an incubation time of 120 h as optimal for maximum lactose conversion to lactic acid.

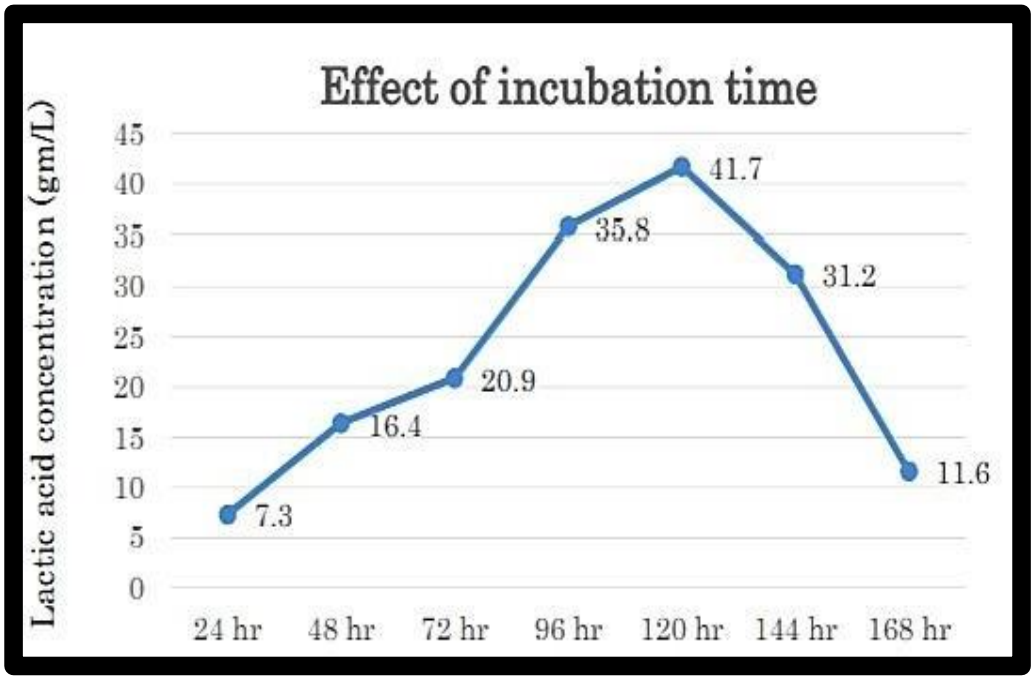
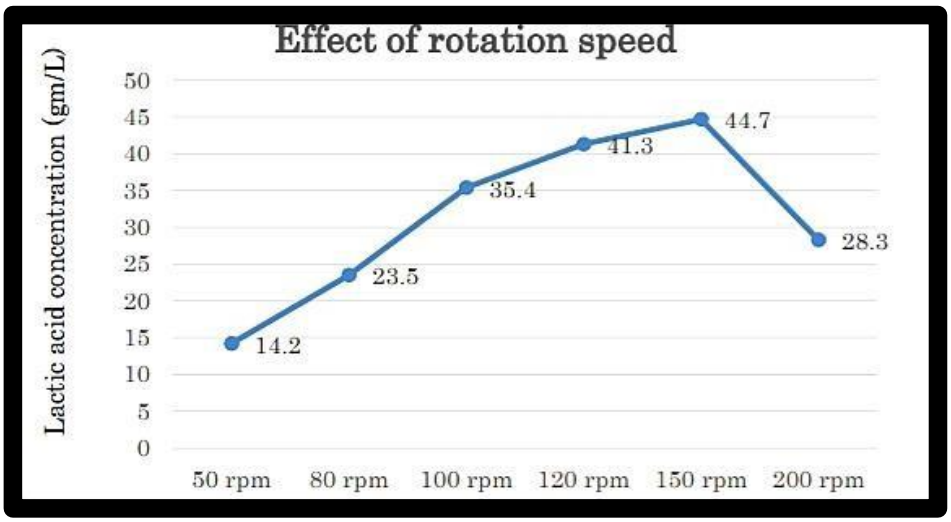


Figure 7: Effect of incubation time on lactic acid production

Effect of rotation speed

To find out optimal rotating speed in shaker incubator for the maximal lactose utilization and lactic acid production, the whey medium inoculated with bacterial culture was incubated for different rpm ranging from 50-200 (Figure 5). We found maximum production of lactic acid at 150 rpm when other parameter kept optimum. When rpm increase to 200, the quantity reduced [20,34-39]. So, it is proved that along with all other optimum parameter shaking speed also influence in lactose utilization and lactic acid production by Lactobacillus sp.

Figure 8: Effect of rotation speed on lactic acid production



CONCLUSION

These bacteria were isolated and identified on the basis of Biochemical and morphological characteristics from curd sample and screened for lactic acid production and lactic acid production parameters were optimised for these isolates using various media components. L. acidophilus was found to be prevalent in dahi Inoculum size. Incubation time and shaking speed were optimized to enhance the conversion of whey sugar into lactic acid. The optimum condition was found for fermentation with the process condition of PH 6.5. temp 37°c &

Inoculum size 4%. (v/v) with an incubate of 120h & effective rotation speed of 150. The above mentioned optimizes process parameters can be used. In conclusion, Lactic acid is one of the most important chemical that can be derived from various waste products. Curd is the by-product of milk, which can be used for lactic acid production. The data presented in this work supports the use of curd for valuable lactic acid production under presented optimized condition.

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