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Fermentative study on optimization of lactic acid production from cane sugar by *Lactobacillus* spp.

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Abstract
The fermentative study on optimization of lactic acid production from cane sugar molasses by isolated Lactobacillus bacteria have been carried out to assess the most suitable condition for its production and to obtain high yield. The study on the optimization effect of different process parameters such as pH of the medium, temperature, inoculums size, incubation time and agitation rate were done in order to enhance the conversion of cane sugar into lactic acid. Optimization was done for Initial pH, temperature, inoculum size, incubation time and agitation rate. The optimum condition was found for fermentation with the process conditions of pH (8.0) temperature (40 °C) and inoculum size (7% (v/v)) with an incubation time of 144 h and effective agitation speed of 175. The above mentioned optimized process parameters can be used in large scale production of lactic acid fermentation in further investigations by using cane sugar as a substrate .
Keywords: Fermentation, lactic acid, cane sugar molasses, Lactobacillus, optimization conditions.

Introduction
Lactic acid is a natural organic acid and found to have variety of applications in pharmaceutical, chemical, food and health care industries. It is a very important product and has got importance in pharmaceutical preparation, leather industry, and electroplating and pork industry [1-3]. It is also used in production of polypropylene oxide, polypropylene glycol, biodegradable poly lactic acid, and acrylic fibers [4]. Production of lactic acid by submerged fermentation is a sound and viable method [5-7]. The fermentation process involves production of lactic acid in short duration with assured production. It is one of the most ancient products of microbial origin. Recently there has been a great interest in lactic acid production from biomass via fermentation [8].
Lactic acid can be produced by both fermentative and chemical synthesis. The production of lactic acid by fermentation process has been an interest due to its advantages as chemical synthesis produces isomers (L(+) or D(-) of lactic acid. Literature survey revealed that commercially, lactic acid is manufactured by synthetic as well as fermentation methods by many author[9-18]. These methods use renewable carbon sources while synthetic route depends upon non-renewable petrochemicals. Thus, fermentation process is more attractive for country like India which has poor petrochemical resources. There exist so many fermentation routes for the production of lactic acid. Production of lactic acid by fermentations compared to chemical method from renewable resources is more economical route. The success of the fermentation in the production of various chemicals by selected microorganism to give high yield of the desired product in a reasonable time from cheap and readily available raw materials [19-27].

Molasses of cane sugar is a by-product of sugar industry used as a raw material for lactic acid fermentation. *L.delbrueckii* is one of the potent microorganisms reported to utilize molasses[28].

On a commercial scale, lactic acid is commonly manufactured by batch fermentation. Monteagudo et.al [29] studied the effect of fermentation parameters (temperature, pH, inoculum and initial sugar concentration) on lactic acid production from beet molasses by *Lactobacillus delbrueckii*. The fermentation process proved several advantages, including easy scale up, high substrate utilization, less chances of contamination This process has also proves to be easy downstream processing and most importantly less time requirement for metabolites production[30-32].

Presently, almost all lactic acid production worldwide from various resources comes from the fermentative route[33] . Recently Diptendu Sarkar etal. studied on the effect of different process parameters such as pH of the medium, temperature, inoculums size, incubation time and shaking speed were optimized to enhance the production lactic acid[34]. Keeping this in view, the wide range of applications and utilities of lactic acid and its derivative prompted us to undertake a microbial study on the optimization of lactic acid production from cane sugar by *Lactobacillus bacteria*.

MATERIALS AND METHODS

i. Maintenance of *Lactobacillus* culture and inoculum preparation

The culture of Lactobacillus acidophilus was maintained on MRS media agar plate with sub culturing at regular intervals during the study. The culture master copy was stored at −4°C and inoculum preparation was done by process briefly described here, the culture was activated by inoculation in fresh MRS liquid medium and incubation at 37°C for 48 hours. 2.0 ml of inoculum was taken from this broth and added to the freshly prepared MRS broth (48 ml). This was then incubated for 48 hours at 37°C at shaker incubator with speed set at 200 rpm.

ii. Substrate

The molasses used for the study was purchased from local vendors and then diluted in distilled water before using in the experiment.

iii. Pretreatment of cane molasses

Pretreatment of Cane molasses was done prior to using it in the fermentation process for lactic acid. For this the cane molasses solution was boiled with H_2SO_4 (1N) (1 Lit molassess+35ml H_2SO_4). Boiling was done for 30 min, then mixture was allowed to cool. This mixture was then neutralized using 3 % CaO. Later, this was left to stand for clarification (kept for overnight).

The supernatant was then treated with activated charcoal in equal proportion (1:1) for 2 h to obtain the appropriate opacity and remove other interfering compounds.

iv. Fermentation media

The fermentation media was prepared based on the composition given in Table1.

Table 1. Ingradients composition of fermentation medium.

MRS Agar Media Ingredients	**Gms / Litre**
Proteose peptone	10.000
Peptone	10.000
Yeast extract	05.000
Dextrose (Glucose)	20.000
Tween 80 (Polysorbate 80)	01.000
Ammonium citrate	02.000
Sodium acetate	05.000
Magnesium sulphate	00.100
Manganese sulphate	00.050
Dipotassium hydrogen phosphate	02.000
Agar	12.000
Final pH (at 25°C)	6.5±0.2

MRS Broth Ingredients	Gms / Litre
Proteose peptone	10.000
Peptone	10.000
Yeast extract	05.000
Dextrose (Glucose)	20.000
Tween 80 (Polysorbate 80)	01.000
Ammonium citrate	02.000
Sodium acetate	05.000
Magnesium sulphate	00.100
Manganese sulphate	00.050
Dipotassium hydrogen phosphate	02.000
Final pH (at 25°C)	6.5±0.2

Agar.

Fermentation Media Ingredients	Gms / Litre
Peptone	10.0
Meat extract	10.0
Yeast extract	5.0
Tween-80	1.0
K_2HPO4	2.0
Sodium acetate	5.0
Tri-ammonium citrate	2.0
MgSO4.7H2O	0.2
MnSO4.4H2O	0.05
Substrate (molasses)	2% (Glucose replaced with sugarcane molassess)

3. Optimization of process parameters for lactic acid Production

Lactic acid production using *Lactobacillus acidophilus* with molasses as substrate was optimized at several parameters to assess the most suitable condition for its production and obtain high yield. Optimization was done for initial pH, temperature, inoculum size, and incubation time and agitation rate. Every parameter was optimized for required variants summarized in Table2.

Table 2 optimization of different parameters.

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S.No.	Parameter	Variations in parameters
1	Initial pH of the medium	4, 5 , 6, 7.5, 8 and 9
2	Temperature	25°C, 30°C, 35°C, 40°C, 45°C and 50°C
3	Substrate innoculum size	1%, 2%, 3%, 4%, 5% , 6%, 7% , 8% and 9%
4	Incubation period	24hrs, 48hrs, 72hrs, 96hrs, 120hrs, 144hrs 168hrs 192hrs and 216hrs
5	Agitation rate	50rpm, 75rpm, 100rpm, 125rpm, 150rpm, 175rpm, 200rpm, 225 rpm and 250 rpm

The parameter methodology is described below: .

The experimental set up of batch cultures was done in a 100ml Erlenmeyer flasks with 50ml fermentation media. Optimization of pH was performed with 6 flasks containing 100ml of fermentation media in each flask (adjusted to) and 2% molasses was added, incubation was

done at 37°C at agitation rate of 200 rpm for each flask separately. Reading was recorded after 24 hours to determine the quantity of lactic acid produced[35].

ii. Temperature

The temperature is one of the important factors, which influences the activity of metabolic cell enzymes. These enzymes are most active at optimum temperature. In optimum temperature enzymatic reaction takes place at 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[36] . Krischke et al., studied on the production of lactic acid and found that 37°C temperature was optimum for lactic acid production using *L. casei* [37]. Ilmen et al also reported maximum lactic acid production of 33.72 gm/L at 37°C by L. casei [38]. From the above observations, it is cleared that a temperature range of 37-40°C was considered optimal for the production of lactic acid using bacterial cells. Thus the present authors selected 37^0C to study on optimization of lactic acid production from cane sugar by *lactobacillus.*

Optimization was done for temperature in six different flasks, experiment were conducted with the mentioned composition of fermentation media in 100 ml erlenmeyer flasks containing 50 ml media, these flasks were inoculated with culture, supplemented with 2% molasses and incubation done at 25°C, 30°C, 35°C, 40°C, 45°C and 50°C for each flask separately at agitation rate of 200 rpm. Reading was recorded after 24 hours to determine the quantity of lactic acid produced.

iii. Innoculum size

The experimental set up of batch cultures was done in a 100ml Erlenmeyer flasks with 50ml fermentation media. Optimization of inoculum size was performed with 6 flasks containing 100ml of fermentation media in each flask and 1%, 2%, 3%, 4%, 5% and 6% , 7%, 8% and 9 molasses was added, incubation was done at 37°C at agitation rate of 200 rpm for each flask separately. Reading was recorded after 24 hours to determine the quantity of lactic acid produced.

iv. Incubation time

The experimental set up of batch cultures was done in a 100ml Erlenmeyer flasks with 50ml fermentation media. Optimization of incubation time was performed with 6 flasks containing 100ml of fermentation media in each flask and 2% molasses, incubation was done at 24hrs, 48hrs, 72hrs, 96hrs, 120hrs and 144hrs, 168hrs, 192hrs and 216hrs for each flask separately at agitation rate of 200 rpm. Reading was recorded after 24 hours to determine the quantity of lactic acid produced.

v. Agitation rate

The experimental set up of batch cultures was done in a 100ml Erlenmeyer flasks with 50ml fermentation media. Optimization of agitation rate was performed with 6 flasks containing 100ml of fermentation media in each flask and 2% molasses, incubation was done at 37°C at agitation rate of 50rpm, 75rpm, 100rpm, 125rpm, 150rpm, 175rpm , 200rpm 225rpm and 250rpm for each flask separately. Reading was recorded after 24 hours to determine the quantity of lactic acid produced

Estimation of Lactic acid using Spectrophotometer method

Determination of lactic acid was done using spectrophotometric method. The method is based on principle of detecting the colored product obtained on resultant reaction of lactate ions and iron (III) chloride which absorbs at 390 nm. Thus, pure lactic acid was used as standard to plot a calibration graph (Fig.1) and evaluate the quantity of lactic acid produced in each flask from the standard calibration graph shown in Table3.

Table 3 Calibration graph of standard lactic acid

S.No.	Concentration (g/l)	Absorbance
1	0.5	0.020
2	1.0	0.119
3	2.0	0.237

4	4.0		0.342
5	8.0		0.371
6		10.0	0.486

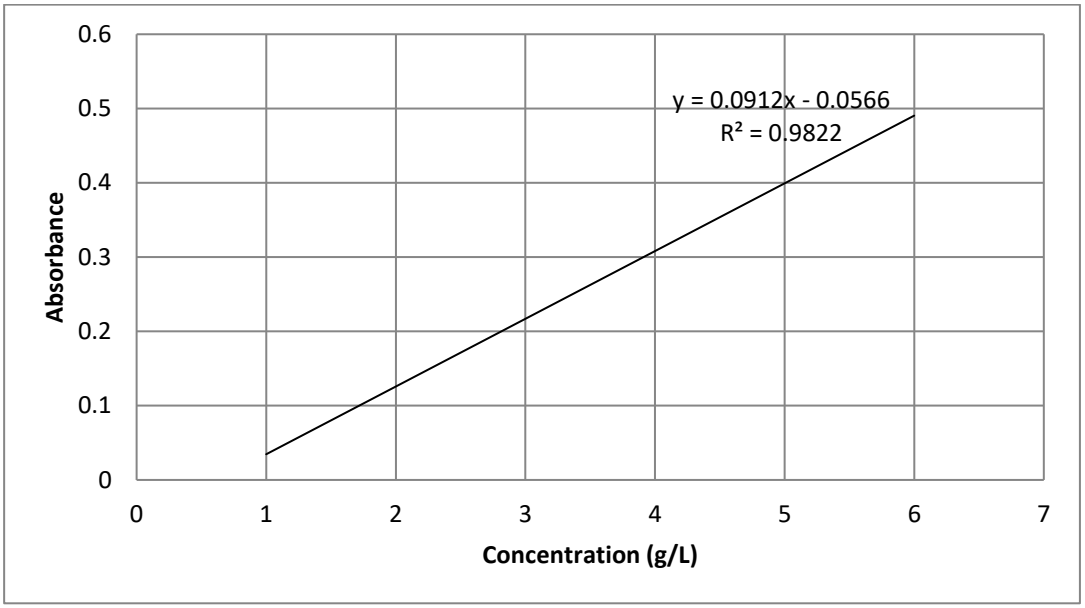


Figure 1 Calibration graph of standard lactic acid

In each condition, sample was taken from the flask, centrifuged at 8,000 rpm for 8 min, pellet was discarded and supernatant used for the estimation of lactic acid. 50 µL of sample (supernatant) was mixed with 2 ml of iron (III) chloride solution (0.2%). The colored product has stability of 15 mins. Therefore, reading could be recorded within this time period.

Results and Discussion

The results of optimization of various parameter for the Lactic acid production using Lactobacillus acid with molasses as substrate to assess the most suitable condition (Initial pH, temperature, inoculum size, incubation time and agitation rate) for obtaining high yield have been incorporated.

Effect of pH on fermentation

The fermentation medium was adjusted to different pH (4.0, 5.0, 6.0, 7.5 and 8.0, 9.0) for optimizing and kept in shaker incubator at 37°C with rotating speed of 200 revolutions per minute. Lactic acid production was checked after 24 h. The results of lactic acid production at varying pH have been incorporated in Table4.

Table 4. optimization of pH on lactic acid production

Optimization of pH S.No.	pH	Absorbance	Concentration of Lactic acid (gm/L)
1	pH- 4.0	0.98	19.29
2	pH- 5.0	1.11	22.02
3	pH- 6.0	1.43	28.73
4	pH- 7.5	1.46	29.36
5	pH- 8.0	1.49	29.99
6	pH- 9.0	1.30	26.00

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 (29.99 gm/l) was obtained at pH 8.0 on 24h of incubation. From pH 4.0 to 6.5 drastically increase the fermentative product, whereas after optimum pH 6.5 to 8.0, the lactic acid production increases to small extent. Thus our findings concluded that a pH 8.0 would be the optimal for maximum

lactic acid production from cane sugar *Lactobacillus* spp. (Shown in fig.2)

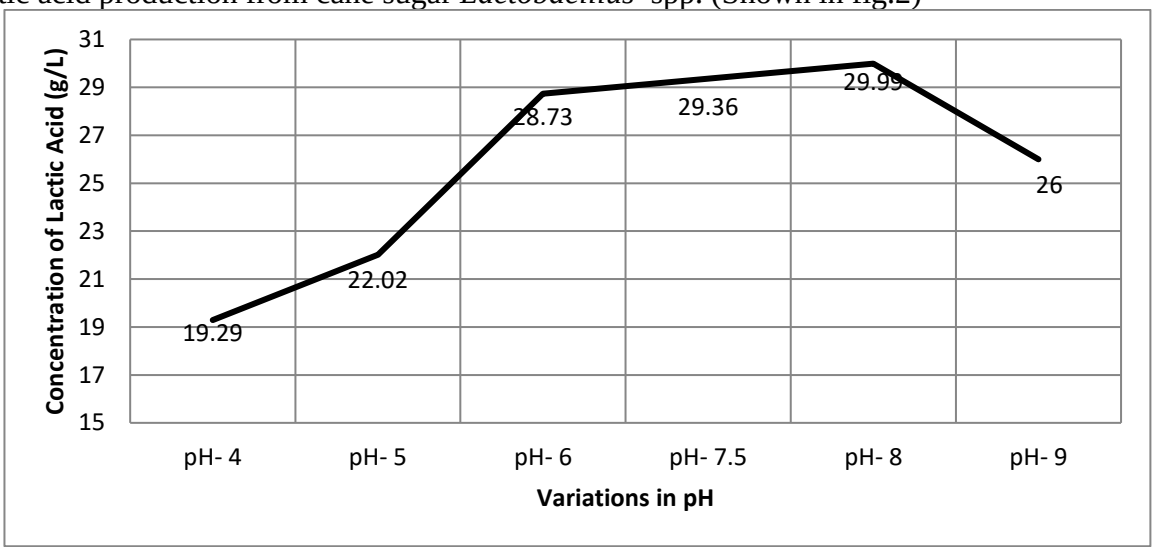


Figure 2 Effect of pH on lactic acid production

Effect of Temperature on fermentation

The optimum pH was maintained at five different temperatures (20, 25, 30, 37, 45 and 50°C) by keeping them with rotating speed of 200 revolutions per minute and the lactic acid production was estimated after 24 h which is summarized in Table5.

Table 5. Optimization of Temperature for Lactic acid production

Optimization of Temperature S.No.	Temperature	Absorbance	Concentration of Lactic acid (gm/L)
1	25°C	0.72	13.84
2	30°C	0.77	14.89
3	35°C	1.04	20.55
4	40°C	1.54	31.04
5	45°C	1.24	24.75
6	50°C	1.05	20.76

To find the optimum temperature for lactic acid production, after adding cane sugar into medium, inoculation was incubated at a temperature range of 25-50°C(Fig. 3). The lactic acid production increased sharply with increase in the temperature from 25°C up to 40°C; and maximum production was found at 40°C(31.04 mg/L)however, an decrease in at 45°C (24.75 gm/L) and much lower of lactic acid p (20.76 mg/L) production was found at 50°C.

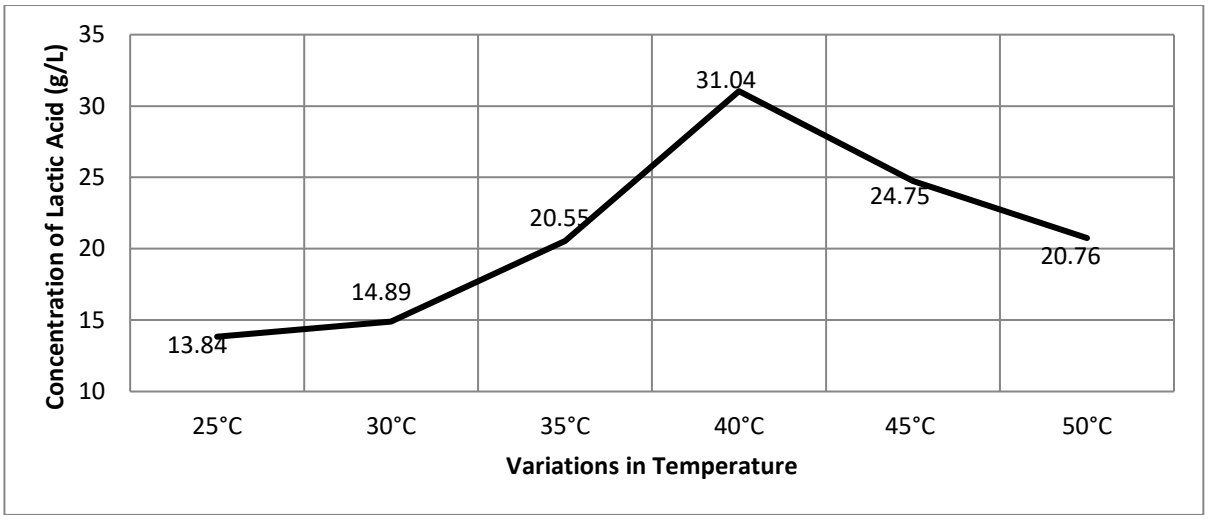


Figure 3 Optimization of Temperature for Lactic acid production

Effect of inoculum size on fermentation

To study the influence of inoculum concentration on the lactic acid fermentation, different inoculum concentration (1-9%, v/v) were added sequentially to the fermentation medium and the lactic acid production was measured after 24 h reported in Table6.

Table 6 Optimization of incubation size for lactic acid production

S.No.	Innoculum size	Absorbance	Concentration of Lactic acid (gm/L)
1	1%	0.31	5.25
2	2%	0.55	10.28
3	3%	0.68	13
4	4%	0.97	19.08
5	5%	1.14	22.65
6	6%	1.43	28.73
7	7%	1.48	29.778
8	8%	1.02	20.133
9	9%	0.84	16.36

To find out the influence of inoculum concentration on the lactic acid production, different inoculum levels (1-9%, v/v) were added to the fermentation medium (Figure 3).

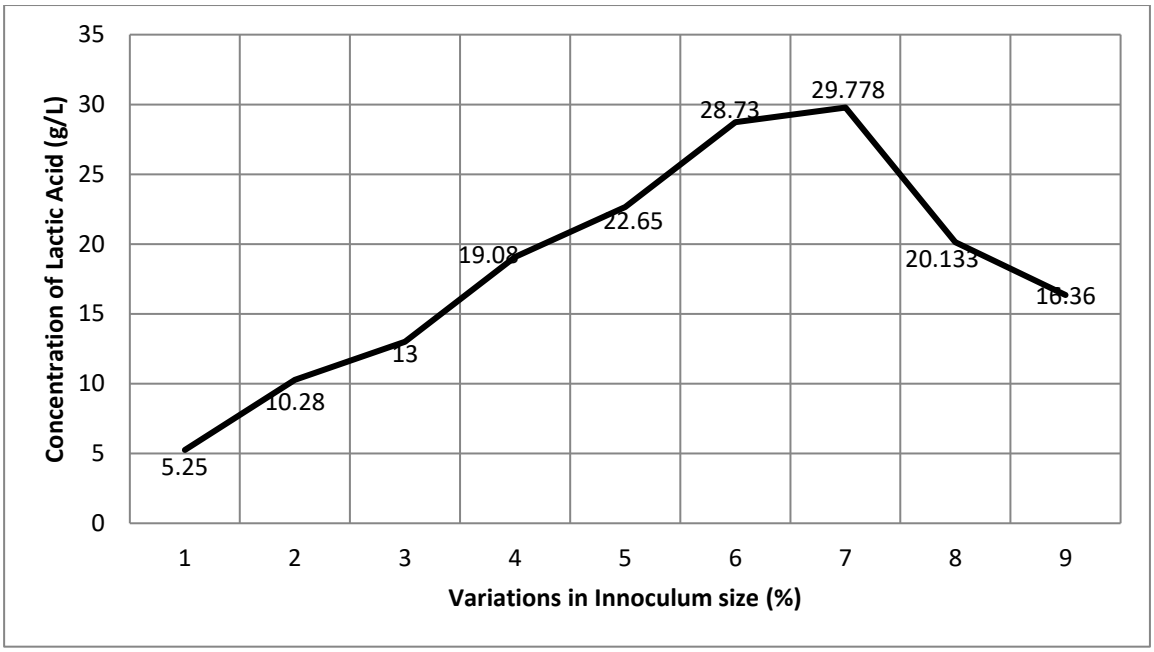


Figure 4 Effect of Innoculum size on lactic acid production

The lactic acid production increased sharply with the rise in inoculum concentration up to !% to 7% (v/v),. The maximum lactic acid production of 29.778 gm/L was observed with the adding of74% (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 (5.25gm/L). From the above observations, an inoculum of 7% (v/v) could be considered optimal for achieving maximum lactic acid production using 24 h old bacterial culture and 7% (v/v) inoculum concentration was used in the subsequent studies.

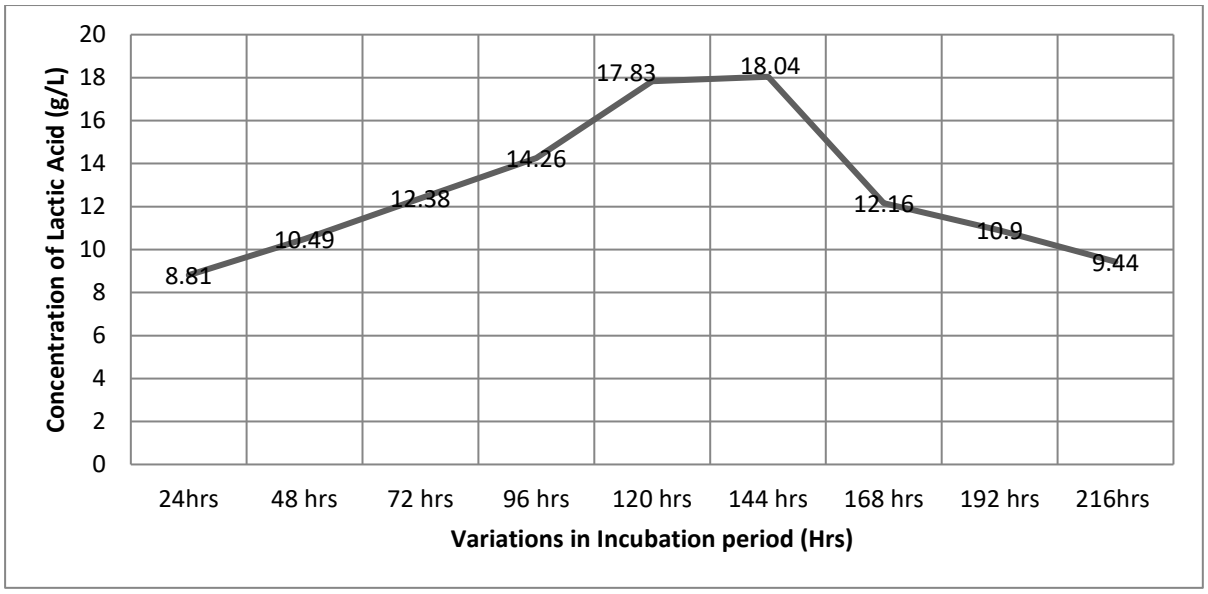
Effect of Incubation Period on fermentation

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, 168h 192hrs and 216hrs respectively under the found above optimized conditions. At the end of each incubation period, lactic acid produced was estimated have been reported in Table7.

Table 7 Optimization of incubation period for lactic acid production

F S.No.	Incubation period	Absorbance	Concentration of Lactic acid (gm/L)
1	24 hrs	0.48	8.81
2	48 hrs	0.56	10.49
3	72 hrs	0.65	12.38
4	96 hrs	0.74	14.26
5	120 hrs	0.91	17.83
6	144 hrs	0.92	18.04
7	168 hrs	0.64	12.16
8	192 hrs	0.58	10.9
9	216hrs	0.51	9.44

Figure 5. Optimization of Incubation period for lactic acid production



The optimal incubation time for the maximal lactic acid production, the cane sugar molasses medium inoculated with bacterial culture was incubated for different time at 24, 48, 72, 96, 120, 144 and 168, 192 and 216 hour under the above optimized conditions. The samples were taken out at specified time intervals and the results obtained are presented in Figure. As evident from the results, an increase in lactose utilization and subsequent lactic acid production was found increased till 144 h and thereafter decrease A maximum lactic acid production of 18.04 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 144 hrs has been generally used for lactic acid production using lactobacilli cultures. Thus 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 144 h as optimal for maximum lactic acid production shown in Fig.5.

Effect of agitation rate on fermentation

To study the influence of agitation rate on the lactic acid fermentation, different rpm at (50 to 250) were set sequentially to the fermentation process respectively under the found above optimized conditions and the lactic acid production was measured after 24 h which have been summarized below in Table8..

Table 8 Optimization of agitation rate for lactic acid production

S.No.	Agitation rate	Absorbance	Concentration of Lactic acid (gm/L)
1	50rpm	0.35	6.08
2	75rpm	0.24	3.78
3	100rpm	0.48	8.81
4	125rpm	0.79	15.31
5	150rpm	1.12	22.23
6	175rpm	1.4	28.1
7	200rpm	1.01	19.92
8	225rpm	0.73	14.05
9	250rpm	0.42	7.55

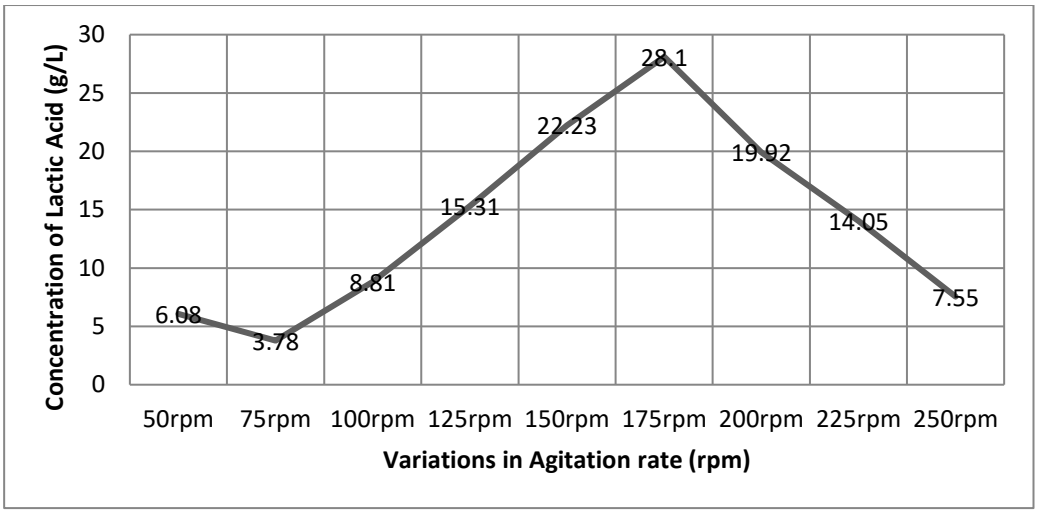


Figure 6. Optimization of Agitation rate for lactic acid production

To find out optimal rotating speed in shaker incubator for the maximal lactic acid production, the canesugar molasses medium inoculated with bacterial culture was incubated for different rpm ranging from 50-250 (Figure). We found maximum production of lactic acid at 175 rpm when other parameter kept optimum. When rpm increase to 200, the quantity reduced (Fig.6) So, it is proved that along with all other optimum parameter shaking speed also influence in lactose utilization and lactic acid production by *Lactobacillus* spp.

Conclusion

The work on the fermentative study on optimization of lactic acid production from cane sugar by lactobacillus concluded that Lactic acid is one of the most important chemical that can be derived from various products. Cane sugar can also be used for lactic acid production. The effect of different process parameters such as pH of the medium, temperature, inoculums size, incubation time and agitation rate were optimized to enhance the conversion of cane sugar into lactic acid. The data presented in this work supports the use of cane sugar for valuable lactic acid production under presented optimized condition.

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