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MICROBIAL LACTIC ACID PRODUCTION: FIELD INSIGHTS AND FERMENTATION OPTIMIZATION

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Abstract: The fermentative study focused on optimizing lactic acid production from cane sugar molasses using isolated Lactobacillus bacteria. The aim was to identify the most favorable conditions for high yield. Various process parameters including pH, temperature, inoculum size, incubation time, and agitation rate were optimized to maximize the conversion of cane sugar into lactic acid. The study determined that the ideal conditions for fermentation were a pH of 8.0, temperature of 40°C, and inoculum size of 7% (v/v), with an incubation time of 144 hours and agitation speed of 175 rpm. These optimized parameters offer potential for large-scale lactic acid fermentation using cane sugar as a substrate. The research underscores the significance of lactic acid as a valuable chemical derived from diverse sources, with cane sugar representing a viable option. Statistical analysis confirmed the significant impact of the optimized factors on lactic acid production. Future investigations could explore scaling up production using these parameters, thus advancing lactic acid fermentation practices.

Keywords: Fermentation, lactic acid, cane sugar molasses, *Lactobacillus*, optimization conditions, ANOVA

1. Introduction: Lactic acid, a versatile compound with applications spanning pharmaceuticals, chemicals, food and more, is predominantly produced through submerged fermentation, offering rapid and reliable yields[1-3]. While both fermentation and chemical synthesis pathways exist, fermentation stands out for its environmental benefits and renewable resource utilization[4-5]. Cane sugar molasses serves as a key substrate for lactic acid fermentation, with Lactobacillus delbrueckii as a potent microorganism[6-8]. Batch fermentation is the preferred method, offering scalability and

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efficiency[9]. Recent studies focus on optimizing fermentation parameters for enhanced production[10]. With its wide-ranging applications, microbial studies targeting optimized lactic acid production from cane sugar hold significant promise[11.]

2. Experimental, materials and methods

Fieldwork was conducted in two phases:

Phase I: During this two-day period, visits were made to various sugarcane-growing villages in the Purulia district of West Bengal, namely Bhelawatar, Sirkabad, Lahabani, Palasbani, and Manjhidih. Discussions were held with local farmers to gather insights into sugarcane cultivation practices, including the optimal stages for growth, suitable climatic conditions, and the timing and location for harvesting sugarcane molasses. Feedback from farmers revealed a decline in interest in sugarcane cultivation due to factors such as high labor costs, expensive fertilizers, and other economic challenges.

Phase II: A team of three members visited the sugarcane fields located in Bhelwatar, Sirkabad, Purulia district, West Bengal. The coordinates for the site were Latitude: 86.17789, Longitude: 23.8035. Observations were made regarding the cutting of sugarcane stalks, which were then transported to the processing unit.

Process: The process of obtaining molasses from sugarcane involved several steps:

I. Harvested sugarcane stalks were transported to the processing unit.
II. The stalks were pressed using a sugarcane juice machine, yielding fresh juice.
III. The obtained juice was transferred to a large iron bowl and boiled for 2-3 hours.
IV. During the boiling process, molasses surfaced on the juice, which was carefully removed and collected.
V. Continuous stirring was maintained during boiling to ensure efficient extraction of molasses.

The process of obtaining molasses from sugarcane shown in Fig1

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Figure 1. The journey from sugarcane to molasses unfolds through a series of meticulous steps

2.1Analysis of lactic acid production

To maintain the Lactobacillus culture, it was periodically subcultured on MRS agar plates throughout the study, with the master copy stored at -4°C. Inoculum preparation involved activating the culture in fresh MRS liquid medium, followed by incubation at 37°C for 48 hours. Subsequently, 2.0 ml of the inoculum was transferred to freshly prepared MRS broth (48 ml), which was then incubated for an additional 48 hours at 37°C with agitation at 200 rpm.

For substrate preparation, molasses obtained from local vendors was diluted in distilled water before use. Prior to fermentation, cane molasses underwent pretreatment by boiling with 1N H_2SO_4 (1 Lit molasses + 35 ml H_2SO_4) for 30 minutes, followed by

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cooling and neutralization with 3% CaO. The mixture was allowed to stand overnight for clarification, then treated with activated charcoal (1:1 ratio) for 2 hours to achieve appropriate opacity and remove interfering compounds.

The fermentation media were prepared according to the composition outlined in Table2 and 3

Table 1. Ingredients composition of fermentation medium.

Ingredient	Gms / Litre
Proteose peptone	10
Peptone	10
Yeast extract	5
Dextrose (Glucose)	20
Tween 80 (Polysorbate 80)	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium hydrogen phosphate	2
Agar	12
Final pH (at 25°C)	6.5±0.2

Table 1. Composition of growth medium (per liter) with molasses as substrate

Ingredient	Gms / Litre
Peptone	10
Meat extract	10
Yeast extract	5
Tween-80	1
K2HPO4	2
Sodium acetate	5
Tri-ammonium citrate	2
MgSO4.7H2O	0.2
MnSO4.4H2O	0.05
Substrate (molasses)	2% (Glucose replaced with sugarcane molasses)

The study optimized the production of lactic acid using Lactobacillus acidophilus and molasses as the substrate. Various parameters, including initial pH, temperature, inoculum size, incubation time, and agitation rate, were systematically adjusted to enhance yield. Each parameter was fine-tuned to specific variants outlined in Table 4.

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Table 2 optimization of different parameters.

S. No.	Parameter	Variations in Parameters
1	Initial pH of the medium	4, 5, 6, 7.5, 8, 8.5, 9, 9.5 and 10
2	Temperature in ^{o}C	25, 30, 35, 40, 45 and 50
3	Substrate Inoculum Size in%	1, 2, 3, 4, 5, 6, 7, 8 and 9
4	Incubation Period in hrs.	24, 48, 72, 96, 120, 144, 168, 192 and 216
5	Agitation Rate in rpm.	50, 75, 100, 125, 150, 175, 200, 225 and 250

The experimental setup involved batch cultures in 100ml Erlenmeyer flasks containing 50ml of fermentation media. Optimization of pH was conducted using 9 flasks, each containing 100ml of media adjusted to the specified pH. Additionally, 2% molasses was added to each flask, followed by incubation at 37°C with agitation at 200 rpm. Lactic acid production was measured after 24 hours to assess the effects of pH variation.

3. Results and Discussion: Lactic acid estimation was carried out via spectrophotometric method, relying on the detection of a colored product formed from the reaction between lactate ions and iron (III) chloride, with absorption at 390 nm.

In each experimental condition, a sample was extracted from the flask and centrifuged at 8,000 rpm for 8 minutes to remove the pellet. The supernatant was then utilized for lactic acid estimation. A volume of 50 µL of the supernatant was mixed with 2 ml of iron (III) chloride solution (0.2%). The resulting colored product remained stable for 15 minutes, allowing for the recording of readings within this timeframe.

The results obtained from the optimization of various parameters for lactic acid production using Lactobacillus spp. with molasses as a substrate, including initial pH, temperature, inoculum size, incubation time, and agitation rate, have been compiled and integrated.

3.1. **pH** The effect of pH on fermentation was investigated by adjusting the fermentation medium to different pH levels (4.0, 5.0, 6.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10) for optimization. The medium was then placed in a shaker incubator set at 37°C with a rotating speed of 200 revolutions per minute. After 24 hours, lactic acid production was assessed. lactic acid production at varying pH have been incorporated in Table 4.

Table 3. pH Optimization for Lactic Acid Production

	Lactic acid production (g/L)		
pH	C1	C2	Average
4	19.29	19.87	19.58
5	22.02	21.98	22
6	28.73	29.45	29.09
7.5	29.36	29.55	29.455
8	29.99	29.32	29.655

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8.5	28.08	27.76	27.92
9	26.98	26.55	26.765
9.5	24.54	24.02	24.28
10	22.23	21.99	22.11

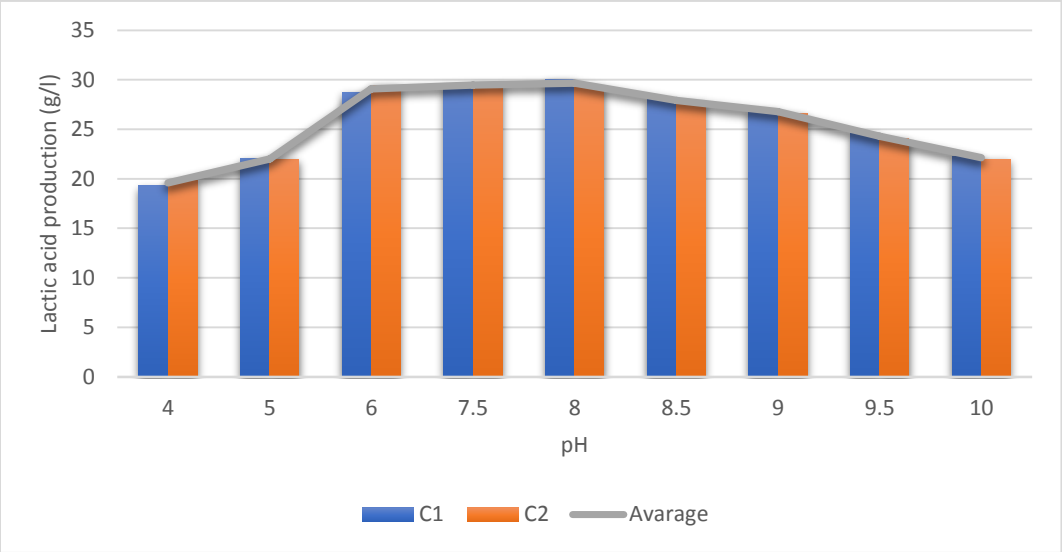

Figure 2. The Influence of pH on Fermentation Optimization

3.2. Temperature

Temperature significantly influences the activity of metabolic enzymes within cells, with optimal temperatures maximizing enzymatic reaction rates. Deviating from the optimal temperature can slow down reaction rates and affect cellular metabolism. For lactic acid bacteria, the optimal growth temperature typically ranges from 20 to 45°C, varying among different species. Studies by Krischke et al. and Ilmen et al. have highlighted 37°C as optimal for lactic acid production using L. casei, with maximum yields observed at this temperature.

Given these findings, a temperature range of 37-40°C was deemed optimal for lactic acid production using bacterial cells. Therefore, the present study selected 37°C to investigate the optimization of lactic acid production from cane sugar by Lactobacillus.

Optimization experiments were conducted in six separate flasks, each containing 100 ml Erlenmeyer flasks with 50 ml of fermentation media. The flasks were inoculated with culture, supplemented with 2% molasses, and incubated at temperatures ranging from 25°C to 50°C, with agitation at 200 rpm. Lactic acid quantities were measured after 24 hours to assess production levels.

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Table 4 Temperature Optimization for Lactic Acid Production

	Lactic acid production (g/L)		
Temperature in ^{o}C	C1	C2	Average
25	13.84	13.22	13.53
30	14.89	14.01	14.45
35	20.55	19.99	20.27
40	31.04	32.01	31.525
45	24.75	25.37	25.06
50	20.76	21.01	20.885
55	18.34	18.55	18.445
60	16.24	16.65	16.445
65°C	15.33	15.69	15.51

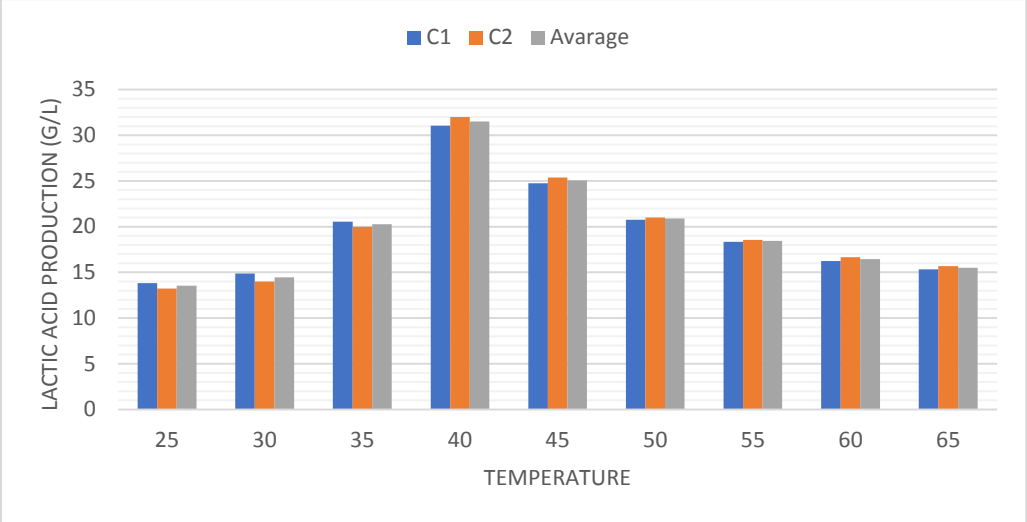

Figure 3. The Influence of temperature on Fermentation Optimization

3.3. Innoculum size: Batch cultures were established in 100ml Erlenmeyer flasks containing 50ml of fermentation media. To optimize inoculum size, six flasks were utilized, each containing 100ml of fermentation media with varying inoculum sizes ranging from 1% to 9%. Additionally, molasses concentrations were adjusted accordingly. Incubation was conducted at 37°C with agitation at 200 rpm for each flask individually. Lactic acid quantities were measured after 24 hours to assess production levels.

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Table 5. Innoculum size Optimization for Lactic Acid Production

	Lactic acid production (g/L)		
Innoculum size	C1	C2	Avg.
0.01	5.25	5.04	5.145
0.02	10.28	10.97	10.625
0.03	13	13.42	13.21
0.04	19.08	19.67	19.375
0.05	22.65	22.98	22.815
0.06	28.73	28.34	28.535
0.07	29.778	29.54	29.659
0.08	20.133	20.32	20.2265
0.09	16.36	16.44	16.4

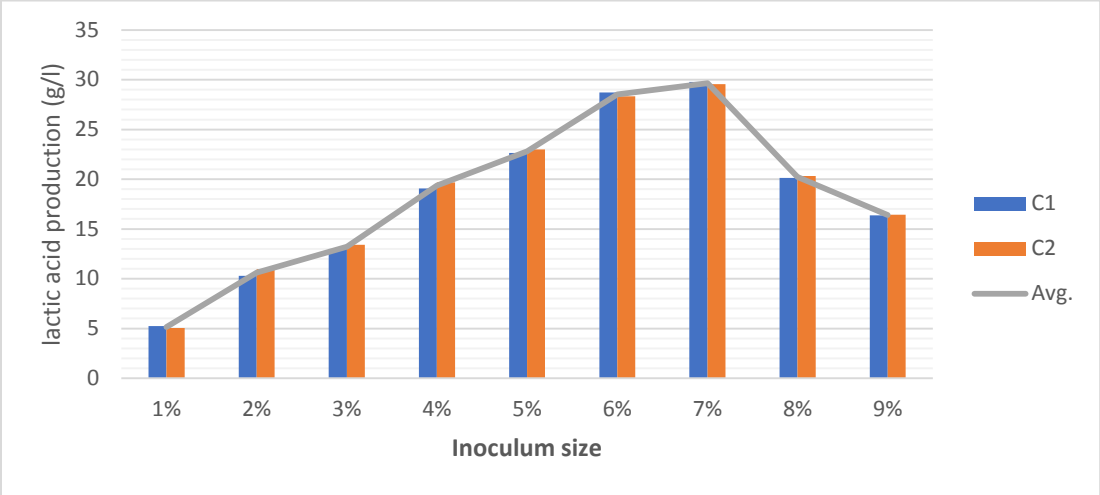

Figure 4. The Influence of inoculum size on Fermentation Optimization

3.4. Incubation time Batch cultures were established in 100ml Erlenmeyer flasks containing 50ml of fermentation media. To optimize incubation time, six flasks were prepared, each containing 100ml of fermentation media supplemented with 2% molasses. Incubation was conducted individually for each flask at time intervals ranging from 24 to 216 hours, including 24hrs, 48hrs, 72hrs, 96hrs, 120hrs, 144hrs, 168hrs, 192hrs, and 216hrs. The agitation rate was maintained at 200 rpm throughout the incubation period. Lactic acid quantities were measured after 24 hours to evaluate production levels.

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Table 6 Incubation time Optimization for Lactic Acid Production

Incubation period in hrs.	Lactic acid production g/L) C1	C2	Average
24	8.81	8.23	8.52
48	10.49	10.02	10.255
72	12.38	12.98	12.68
96	14.26	14.03	14.145
120	17.83	17.24	17.535
144	18.04	17.99	18.015
168	12.16	12.35	12.255
192	10.9	10.08	10.49
216	9.44	9.65	9.545

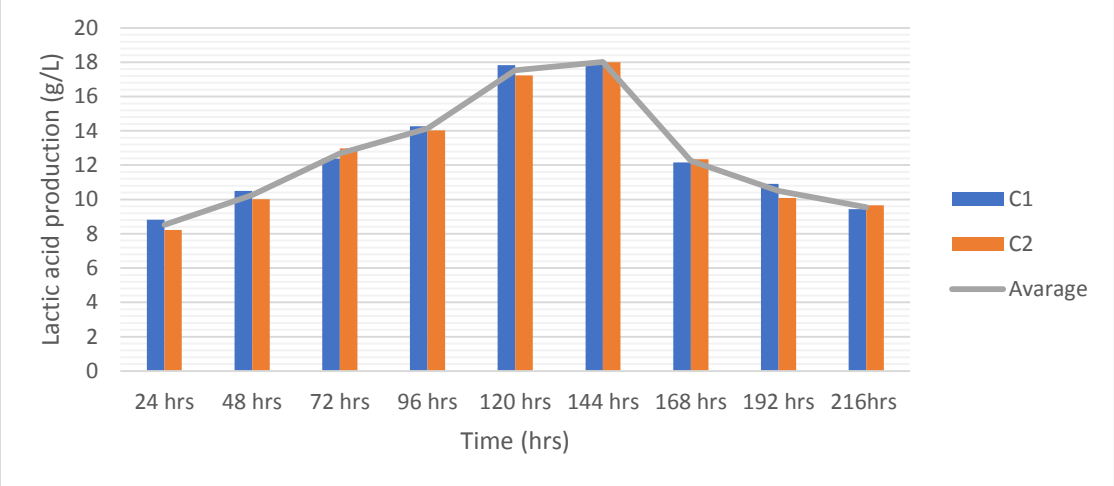

Figure 5. The Influence of incubation period on Fermentation Optimization

3.5. Agitation rate: Batch cultures were established in 100ml Erlenmeyer flasks with 50ml of fermentation media. To optimize the agitation rate, six flasks were prepared, each containing 100ml of fermentation media supplemented with 2% molasses. Incubation was conducted at 37°C with agitation rates ranging from 50rpm to 250rpm, including 50rpm, 75rpm, 100rpm, 125rpm, 150rpm, 175rpm, 200rpm, 225rpm, and 250rpm, for each flask individually. After 24 hours, readings were taken to determine the quantity of lactic acid produced.

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Table 7. Agitation Rate Optimization for Lactic Acid Production

	Lactic acid production (g/L)		
Agitation Rate in rpm	C1	C2	Avarage
50	6.08	5.96	6.02
75	3.78	3.32	3.55
100	8.81	7.98	8.395
125	15.31	16.03	15.67
150	22.23	21.88	22.055
175	28.1	27.77	27.935
200	19.92	19.92	19.92
225	14.05	13.63	13.84
250	7.55	7.98	7.765

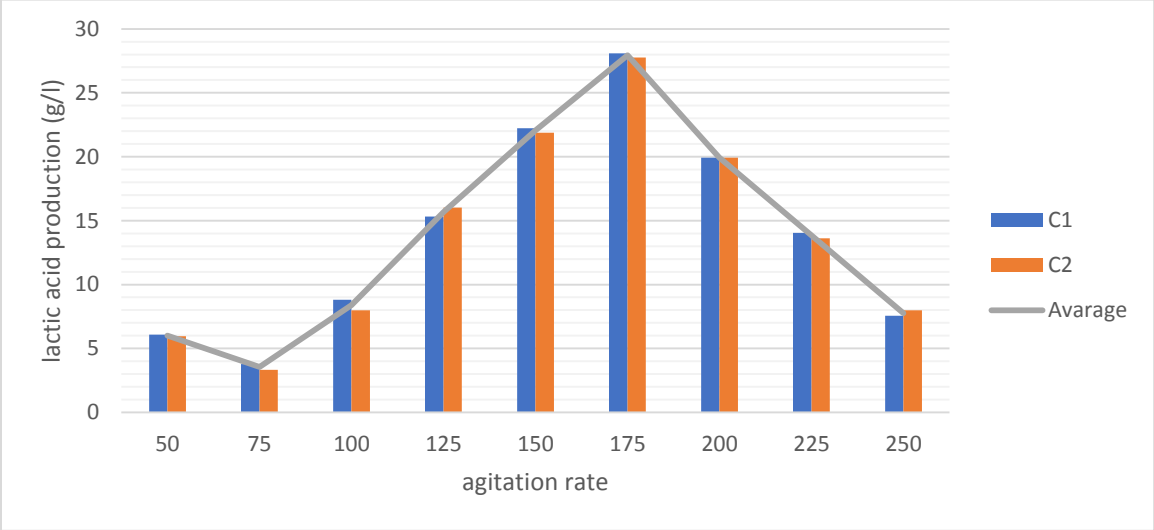

Figure 6. The Influence of agitation rate on Fermentation Optimization.

4. Results of ANOVA calculations: Based on the ANOVA results obtained for optimizing lactic acid production, the hypotheses can be articulated as follows:

Null Hypothesis (H0): There exists no substantial variance in lactic acid production across the examined conditions, implying no discernible impact of pH, temperature, agitation rate, incubation period, or inoculum size.

Alternative Hypothesis (H1): A notable disparity in lactic acid production exists among the tested conditions, indicating the influence of at least one factor—pH, temperature, agitation rate, incubation period, or inoculum size—on lactic acid yield.

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Table 8. Summary of ANOVA Results for Lactic Acid Production Optimization

Source	Degrees of freedom (DF)	Sum of Squares (SS)	Mean Square(MS)	F-Stat	p-Value	F-Critical
Between Groups	4	963.5433	240.8858			
Within Groups	40	1531.2886	38.2822	6.2924	0.0005	2.60597
Total:	44	2494.8319				

A lower p-value means stronger evidence against the null hypothesis, indicating that at least one factor significantly influences the response variable. In this case, the F-value (6.29234) exceeds the critical F-value (2.60597), and the p-value (0.005) is below the usual significance level (0.05). These findings indicate that the model is statistically significant, and at least one factor among pH, temperature, agitation rate, incubation period, and inoculum size affects lactic acid production significantly.

5. CONCLUSION: The Fieldwork in Purulia district, West Bengal, provided insights into challenges facing sugarcane farming, such as rising costs and traditional molasses production methods. Interactions with locals highlighted socioeconomic dynamics and community engagement in agriculture. The fermentative study aimed to optimize lactic acid production from cane sugar by Lactobacillus bacteria, showcasing its versatility and significance. Utilizing cane sugar as a substrate enhanced conversion efficiency, validated by statistical analysis. Future research may explore large-scale production using optimized parameters, advancing lactic acid fermentation. This research not only contributes to understanding lactic acid production but also promotes sustainable production methods in the chemical industry. Based on the provided data and statistical analysis, an experiment was conducted to optimize conditions for microbial lactic acid production. The ANOVA results indicate significant differences between tested conditions, suggesting pH, temperature, agitation rate, incubation period, and inoculum size all impact lactic acid yield.

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