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9. Parametric Determination of Lactobacillus Bulgaricus NCIM-2359

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ABSTRACT:

"Optimization of parameters" deals with selection of lactic acid producing bacteria, selection of cheapest and easily available economic raw material, optimization of concentration of raw material selected, optimization of temperature, pH and incubation period of lactic acid fermentation process. In this paper it has been found that lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 proceeds best when a molasses solution (raw material) 20% (w/v) is allowed to ferment for 6 days of incubation period at 38°C by maintaining the pH value of the fermentation medium to 6.1 along with other nutritional supplements required by the lactic acid bacteria.

KEYWORD:

Lactic acid fermentation, fermentation, Lactobacillus bulgaricus, NCIM-2359

Introduction:

Organic acids, widely used in the food, pharmaceutical and chemical industries, are important chemicals. Fermentation technology for the production of organic acids in particular has been known for more than a century and acids have been produced in aqueous solutions. The development of lactic acid fermentation, with environmental impacts (production of polylactate biodegradable plastics) is welcome in our days. Many studies are focused on how to obtain pure lactic acid. Lactic acid fermentation has been gaining increased attention in the recent years primarily due to its importance as a building block in the manufacture of biodegradable plastics. Lactic acid can be produced from various substrates such as whey permeate, starch hydrolysates which are sources of lactose and glucose respectively. Lactic acid can be manufactured by either chemical synthesis or renewable carbohydrate fermentation. An optimization of parameter (or a decision variable, in the terms of optimization) is a model parameter to be optimized.

During the optimization process, the parameter's value is changed in accordance to its type within an interval, specified by lower and upper bounds. The goal of the optimization process is to find the parameter values that result in a maximum or minimum of a function called the objective function Objective function is a mathematical expression describing a relationship of the optimization parameters or the result of an operation (such as simulation) that uses the optimization parameters as inputs. The optimization objective is the objective function plus optimization criterion. The latter determines whether the goal of the optimization is to minimize or maximize the value of the objective function. Parametric studies on production of lactic acid from molasses fermentation by *Lactobacillus bulgaricus* NCIM-2359 is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation.

A poor selection of medium components can effect cellular growth and little if any yield of fermentation products. The optimization of parameters like concentration of selected raw material, hydrogen ion concentration, temperature and incubation period of the fermentation medium can partially or fully influence the types and ratios of products from among those for which a microorganism has biosynthetic capability[12-15]. Thus, optimization of parameters for production of lactic acid from molasses fermentation by Lactobacillus *bulgaricus* NCIM-2359 is very important and critical. All organisms require source of energy for their metabolism. Some organisms can use reduced inorganic compounds as electron donors while other organisms use organic compounds as electron donor. From this brief excursion into the nutritional requirement of bacteria, it is apparent that to grow bacteria successfully the laboratory worker must provide the proper and appropriate kind of medium and also an appropriate set of physical condition such as temperature, incubation period, and pH etc. Thus, by understanding the various physico-chemical parameters controlling enzyme catalysed activities of different microbes, specially lactic acid bacteria *Lactobacillus* bulgaricus NCIM-2359, the biological activity may be increased, decreased, or destroyed partially or completely. Among the significant physico-chemical parameters for submerged fermentative production of lactic acid by *Lactobacillus bulgaricu*s NCIM-2359 are the selection of substrate raw material and its percent dilution concentration, H^+ ion concentration (pH) of the medium, temperature and incubation period. Indeed, enzymes are very sensitive to elevated temperatures and other fermentation parameters. Because of the protein nature of an enzyme thermal denaturation of the enzyme protein with increasing temperatures will decrease the effective concentration of an enzyme and consequently decrease the reaction rate. Thus, on increasing the temperature enzyme activity gradually increases, but at certain stages temperature inactivates the rate of reaction and finally enzyme is denatured (at high temperature inactivates the rate of reaction and finally enzyme is denatured (at high temperature) as it is proteinaceous in nature [1-11]

There is a high chemical affinity of the substrate for certain areas of the enzyme surface called the active site. The active site on the enzyme surface is actually a very small area, which means the large regions of the enzyme protein (which has hundreds of amino acids) do not contribute to enzyme specificity or enzyme action. It should also be emphasized that the "fit" between an active site of the enzyme surface and the substrate is not a static one; rather it is dynamic interaction in which the substrate induces a structural change in the enzyme molecule, as a hand changes the shape of a glove. Among the conditions affecting the activity of enzymes [13-16] are the following:

(1) Concentration of enzymes
(2) Concentration of substrate
(3) pH and
(4) Temperature

It is obvious that the deviations from the optimal conditions result in significant reduction of enzyme activity. This is characteristic of all enzymes. Extreme variations in pH can even destroy, as can high temperatures; boiling for a few minutes will denature (destroy) most enzymes. Extremely low temperature for all practical purposes, stop enzyme activity but do not destroy the enzymes. Many enzymes can be preserved by holding them at temperatures around 0^0C or lower. Optimum conditions must be estimated in terms of what is based for the entire cell.

Methods and Materials:

Experimental

Medium:

The composition of the production medium for each fermentor flask containing 100 mL production medium is as below:

Molasses : 20%,, Malt Extract : 0.60% $(NH_4)_2HPO_4$: 0.60%,

Yeast extract : 0.60%, $CaCO_3$: 8%, pH : 6.1

Culture medium

Glucose	: 0.6%, Lactose: 0.6%
Sodium-Acetate	: 500.00 mgs
Liver-Extract	: 500.00 mgs
Peptone	: 500.00 mgs Salt Solution A: 0.5ml
Salt solution B	: 0.5ml, pH :5.5-6.1
Sterilization	: 15lbs for 30 minutes
Sub-culture	: Once a month

Requisite amount of distilled water was added to make the total volume 100 ml.**Preparation of Salt Solution A**

It was prepared by mixing the following with water:

KH_2PO_4 : 25.00 g; K_2HPO_4 : 25.00 g

Requisite amount of distilled water was added to make the volume 250 ml.

Preparation of Salt solution B:

It was prepared by mixing the following with water:

$MgSO_4.7H_2O$: 10.00 g, NaCl :500.00 mgs

$MnSO_4.5H_2O$:500.00 mgs $FeSO_4.7H_2O$:500.00 mgs

The above amount were dissolved in requisite amount of distilled water to make up the volume up to 250 ml.

The volume of the culture medium was taken in a dry and clean flask and was plugged with non-absorbent cotton pieces. About 12 clean and dry culture tubes were similarly plugged with non-absorbent cotton. These culture tubes and culture medium were sterilized in an autoclave at 1.71 Kg/I Cm^2 steam pressure for 30 minutes. For solid growth medium 1.5 to 2.0 % agar-agar was added in this solution before sterilization. After cooling, 5.0 ml of the culture medium from the conical flask was transferred to each culture tube. The culture tubes were then allowed to stand in slant position overnight.

Sterilization:

The growth and production media were sterilized in an autoclave maintained at 15 lbs steam pressure for 30 min.

- **Strain:** *Lactobacillus bulgaricus* NCIM-2359 was used in the present study. The strain was procured from NCL, Pune, India.
- **Assay methods:**
- Evalution of lactic acid formed and molasses left unfermented was made colorimetrically[200-201]
- **Age of the inoculum:** 50 hours old.
- **Quantum of the inoculum:** 0.5 ml bacterial suspension of *Lactobacillus bulgaricus* NCIM-2359
- **Molasses concentration:** 2%, 4%, 6%, 8%, 10%, 15%, 20%*, 25%, 30% and 35%
- **Temperature** (in^{0}C): 10, 20, 30, 32,33, 35, 38*, 40, 45, 50, 52, 54, 56, 58 and 60^{0}C
- **Incubation period:** 1, 2, 3, 6*,9, 11, 13, 15, 16, 17,18, 19, 20, 21and 22 days
- **pH:** 5.2, 5.4, 5.8, 6.0, 6.1*, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8 and 6.9

Results and Discussion:

The results of colorimetric analysis are given in the Table 1-6. The value reported are mean of three trials in each case.

Table - 1

Study of the effect of different carbohydrates on lactic acid fermentation by Lactobacillus *bulgaricus* NCIM-2359

Sr. No.	Carbohydrates substrates used	Yield of lactic acid* in g/100 ml	Sugar left unfermented g/100 ml.
1	Arabinose	1.4186472	-
2	Rhamnose	0.3942169	-
3	Xylose	0.9067528	-
4	Glucose	8.8170593	-
5	Fructose	6.9360413	-
6	Galactose	4.9817162	-
7	Sorbose	0.5531321	-
8	Lactose	4.9986254	-
9	Sucrose	7.9830156	-
10	Maltose	2.2162190	-
11	Starch	0.2968514	-
12	Inuline	0.4878350	-
13	Dextrine	0.44113977	-
14	Raffinose	2.2016188	-
15	Mannitol	1.5968274	-
16	Molasses** 20% (w/v)	7.9481130	-

* Each value represents mean of three observation;

(1-7) monosaccharides; (8-10)- disaccharides; (11-14) poly saccarides;

(15)- Polyalcohol; S. No. (16) Molasses contains approximately 52%

fermentable sugars.

** Molasses was employed as raw material due to economical cost.

Table 2

Study of the effect of concentration of molasses on lactic acid feremementation by *Lactobacillus bulgaricus* NCIM-2359 in 6 days of incubation period at pH 6.1 and temperature 38^{0}C

Sr. No.	% Concentration of molasses (in g) (W/V)	Yield of lactic acid* in g/100 ml	Sugar left * unfermented in g/100 ml.
1	2%	0.710952	0.1281695
2	4%	1.4160386	0.3068953
3	6%	2.131487	0.6597280
4	8%	2.8861365	1.3097563

5	10%	3.6501386	1.5986204
6.	15%	5.6254136	1.9835912
7	20% **	7.8926957***	2.1065714
8	25%	8.7430267	3.6958362
9	30%	10.0811367	4.5678069
10	35%	****	—

* Each value represents mean of three observations.

** Optimum concentration of molasses

*** Optimum yield of lactic acid

**** Insignificant value

Table 3

Study of the effect of different pH on lactic acid fermentation by *Lactobacillus bulgaricus* NCIM-2359 from molasses (20%) in 6 days of incubation period at temperature 38^{0}C

Sr. No. of sets of flasks	pH	Yield of lactic acid* in g/100 ml.	Molasses left unfermented* in g/100 ml.
1	5.2	4.5498263	5.4491737
2	5.4	4.8459011	5.1439873
3	5.8	6.1819757	3.8079245
4	6.0	7.3841362	2.6086387
5	6.1**	8.2336951***	1.7574215
6	6.3	7.1469590	2.3543298
7	6.4	6.9036182	2.3326210
8	6.5	5.4536518	2.3081422
9	6.6	****	—
10	6.7	****	—
11	6.8	****	—
12	6.9	****	—

* Each value represents mean of three observations.

** Optimum pH value

** Optimum yield of lactic acid.

**** Insignificant yield of lactic acid.

Table 4

Study of the effect of different pH on lactic acid fermentation by *Lactobacillus bulgaricus* NCIM-2359 from molasses (20%) in 6 days of incubation period at temperature 38^{0}C

Sr. No. of sets of flasks	PH	Yield of lactic acid* in g/100 ml.	Molasses left unfermented* in g/100 ml.
1	5.2	4.5498263	5.4491737
2	5.4	4.8459011	5.1439873
3	5.8	6.1819757	3.8079245
4	6.0	7.3841362	2.6086387
5	6.1**	8.2336951***	1.7574215
6	6.3	7.1469590	2.3543298
7	6.4	6.9036182	2.3326210
8	6.5	5.4536518	2.3081422
9	6.6	****	—
10	6.7	****	—
11	6.8	****	—
12	6.9	****	—

* Each value represents mean of three observations.

** Optimum pH value

** Optimum yield of lactic acid.

**** Insignificant yield of lactic acid.

Table -5

Study of the effect of different temperature on lactic acid fermentation by *Lactobacillus bulgaricus* NCIM-2359 from (molasses 20%) in 6 days of incubation period at pH 6.1

Sr. No. of sets of flasks	PH	Yield of lactic acid* in g/100 ml.	Molasses left unfermented* in g/100 ml.
1	10	2.1563112	7.8136814
2	20	3.6569310	6.3271480
3	30	4.9018976	5.0761138
4	32	5.4591301	4.5411395

Sr. No. of sets of flasks	PH	Yield of lactic acid* in g/100 ml.	Molasses left unfermented* in g/100 ml.
5	33	5.7968562	4.1985795
6	35	6.9828950	3.0148138
7	38**	8.1864325***	1.7932015
8	40	7.9535975	1.7803952
9	45	7.6536310	1.7786106
10	50	****	–
11	52	****	–
12	54	****	–
13	56	****	–
14	58	****	–
15	60	****	–

* Each value represents mean of three observations.

** Optimum temperature

*** Optimum yield of lactic acid.

**** Insignificant yield of lactic acid.

Table -6

Study of the effect of different incubation period on lactic acid fermentation by *Lactobacillus bulgaricus* NCIM-2359 from (molasses 20%) at pH 6.1

Sr. No. of sets of flasks	PH	Yield of lactic acid* in g/100 ml.	Molasses left unfermented* in g/100 ml.
1	1	1.1563952	8.8170391
2	2	2.9513263	7.0431958
3	3	5.8241302	4.1708123
4	6**	7.9126123***	2.0861150
5	9	7.45711630	2.5338131
6	11	6.2535759	3.7456241
7	13	****	–
8	15	****	–
9	16	****	–
10	17	****	–

Sr. No. of sets of flasks	PH	Yield of lactic acid* in g/100 ml.	Molasses left unfermented* in g/100 ml.
11	18	****	–
12	19	****	–
13	20	****	–
14	21	****	–
15	22	****	–

* Each value represents mean of three observations.

** Optimum Incubation period

*** Optimum yield of lactic acid.

**** Insignificant yield of lactic acid.

Discussion:

The data recorded in the table-1 shows the study of effect of different carbohydrate material on production of lactic acid by Lactobacillus *bulgaricus* NCIM-2359. From the results it is clear that sugar substrate becomes very smooth and easy as the molecular size and structure configuration of the carbohydrate substrate molecules becomes simple. The monosaccharides, especially glucose and fructose sugars both have been found much fermentable amongst monosaccharides due to the presence of active carbonyl group being common in glucose and fructose (aldehydic and ketonic groups) are easily phosphorelated due to energy conversation of living cells which is fundamental properties of microbes. Galactose exists in both open-chain and cyclic form. The open-chain form has a carbonyl at the end of the chain.Four isomers are cyclic, two of them with a pyranose (six-membered) ring, two with a furanose (five-membered) ring. Galactofuranose occurs in bacteria, fungi and protozoa. Galactose is a monosaccharide. When combined with glucose (monosacccharide), through a dehydration reaction, the result is the disaccharide lactose. The hydrolysis of lactose to glucose and galactose is catalyzed by the enzymes lactase and ß-galactosidase.[12]

The data recorded in table-2 shows the effect of different concentration of sugary raw material, i.e., molasses substrate. The best result has been noted when 20% (w/v) molasses substrate solution has been allowed for production of lactic acid by *Lactobacillus bulgaricus* NCIM-2359. It has been found that lower concentration of molasses did not give significant yield of lactic acid while higher molasses solution has been found to interfere with the bacterial enzyme activity and thereby retards the production of lactic acid by *Lactobacillus bulgaricus* NCIM-2359.

The data recorded in table-3 the influence of different pH on fermetative production of lactic acid by *Lactobacillus bulgaricus* NCIM-2359. Acidic and basic are two extremes that describe a chemical property. Mixing acids and bases can cancel out or neutralize their extreme effects. A substance that is neither acidic nor basic is neutral. This is a rough measure the acidity of a solution. The "p" stands for "potenz" (this means the potential to be) and the "H" stands for Hydrogen. The pH scale measures how acidic or basic a substance is. The pH scale ranges from 0 to 14. A pH of 7 is neutral. A pH less than 7 is acidic. A pH greater than 7 is basic. The pH scale is logarithmic and as a result, each whole pH value below 7 is ten times more acidic than the next higher value. For example, pH 4 is ten times more acidic than pH 5 and 100 times (10 times 10) more acidic than pH 6.0 The same holds true for pH values above 7, each of which is ten times more alkaline (another way to say basic) than the next lower whole value. For example, pH 10 is ten times more alkaline than pH 9 and 100 times (10 times 10) more alkaline than pH 8. Pure water is neutral. But when chemicals are mixed with water, the mixture can become either acidic or basic. Examples of acidic substances are vinegar and lemon juice. Lye, milk of magnesia, and ammonia are examples of basic substances.

The data recorded in table-4 the influence of different temperature on production of lactic acid by *Lactobacillus bulgaricus* NCIM-2359. Temperature changes have profound effects upon living things. Enzyme-catalyzed reactions are especially sensitive to small changes in temperature. It has been found that production of lactic acid increases with increase of temperature from 10°C to 38°C. At lower temperatures, i.e., 10^0C, 20^0C and 30^0C the yield of lactic acid was found to be discourasing. While the yield of lactic acid gradually falls with the rise of temperature, i.e., 40^0C and onwards. The 38^0C temperature has been found most significant, suitable, and effective for maximum production of lactic acid, i.e., 8.1864325 g/100ml by Lactobacillus *bulgaricus* NCIM-2359 and therefore, this temperature, i.e., 38°C was selected and maintained throughout the experiments described in this paper.

The data recorded in the table-5 reveals the influence of different incubation period on production of lactic acid by *Lactobacillus bulgaricus* NCIM-2359. It has been found that conversion of molasses to lactic acid increases with the increase in incubation period from 1 to 6 days and then normally falls. It was also found that usually consumption of molasses corresponded with the yield of lactic acid and in 6 days, 7.9126123g/100 ml of lactic acid has been obtained. No further increase in the yield of lactic acid has been observed with the further increase in the incubation period.

Conclusion:

It may, therefore, be concluded that production of lactic acid by *Lactobacillus bulgaricus* NCIM-2359 proceeds best when a molasses solution of 20% (W/V) is allowed to ferment for 6 days of incubation period at 38°C temperature by maintaining the pH values of fermenting medium at 6.1 along with other bioingredients supplements required by Lactobacillus *bulgaricus* NCIM-2359.

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