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Optimization of Lactic Acid Production in SSF by *Lactobacillus amylovorus* NRRL B-4542 Using Taguchi Methodology

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Lactic acid production parameter optimization using *Lactobacillus amylovorus* NRRL B-4542 was performed using the design of experiments (DOE) available in the form of an orthogonal array and a software for automatic design and analysis of the experiments, both based on Taguchi protocol. Optimal levels of physical parameters and key media components namely temperature, pH, inoculum size, moisture, yeast extract, $MgSO_4 \cdot 7H_2O$, Tween 80, and corn steep liquor (CSL) were determined. Among the physical parameters, temperature contributed higher influence, and among media components, yeast extract, $MgSO_4 \cdot 7H_2O$, and Tween 80 played important roles in the conversion of starch to lactic acid. The expected yield of lactic acid under these optimal conditions was 95.80% and the actual yield at optimum conditions was 93.50%.

Key words: DOE, Lactic acid production, *Lactobacillus amylovorus*, Optimization, Taguchi Methodology

Lactic acid is a natural organic acid with wide applications in pharmaceutical, chemical, food, and health care industries (Abdullatif *et al.* 2002). It is also widely used in biodegradable plastics, meat preservation, and environmentally friendly green solvents (Payot *et al.* 1999). It is primarily produced by chemical synthesis in industries other than biotechnological processes. Because of its great demand, several microorganisms are screened worldwide to determine their abilities to produce lactic acid. Lactic acid producing bacteria (LAB) are well known to produce lactic acid, of which *Lactobacillus acidophilus, L. delbrueckii, L. intermedius, L. plantarum, L. caseii,* and *L. amylovorus* were the most promising strains. Fungi like *Rhizopus sps* (Litchfield, 1996) and genetically engineered yeast cells have also been studied for lactic acid production. (Donilo *et al.* 1999).

Various starchy substrates (Xiaodong *et al.* 1997), cellulosic substrates (Venkatesh, 1997), like corn straw (Xuexia *et al.* 2001), processed wood (Ana *et al.* 2001), and alfalfa fiber (Hassan *et al.* 2001), have been used for lactic acid production. In the present study, we used raw corn starch for lactic acid production.

Solid Substrate Fermentation (SSF) has proved its supe-

riority over submerged fermentation technology in many ways, including less water required, easy scale up, high substrate utilization, easy elimination of contamination, easy down streaming processing, and cost effectiveness (Pandey, 1992; Krishna and Padma, 1998; Pandey *et al.* 1999; Xuexia *et al.* 2001; Soccol *et al.* 2003).

It has been reported that lactic acid production is dependent on various process variables, including sucrose, yeast extract, $CaCO_3$ (Kotzamanidis *et al.* 2002), and some types of bacterial species. In another study (Sudip *et al.* 2001), optimal control of starch additions in fed-batch processes caused improved performance of the simultaneous saccharification and fermentation of starch to lactic acid. Payot *et al.* (1999) reported that *Bacillus coagulans* growth depends on aeration and nitrogen supplementation, temperature and pH, the initial concentration of sugar affects the fermentation process for lactic acid production. These studies demonstrated that optimization of media components are important for lactic acid production.

Taguchi's method is based upon an approach, which is completely different from the conventional practices of quality engineering. This methodology emphasizes integrating quality into products and processes, whereas usual practice relies upon inspection (Taguchi, 1986). Taguchi essentially utilizes the conventional statistical tools, but simplifies them by identifying a set of stringent guidelines

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for experiment layout and analysis of results. Taguchi's standardized version of DOE, popularly known as the Taguchi method or Taguchi approach, was introduced in USA in the early 1980's (Ross, 1996).

The application of DOE requires careful planning, prudent layout of the experiment, and expert analysis of results (Roy 1990). Based on years of research and applications, Taguchi has standardized methods for each of these DOE application steps. Thus, DOE using Taguchi approach has become a much more attractive tool for practicing engineers and scientists (Roy, 2001).

This Taguchi method not only provides aid for considerable time and cost-saving, but also leads to a more fully developed process. It has several design arrays, such as OA12, OA 18, OA 36, and OA 54, which enable a focus on main effects and help to increase the efficiency and reproducibility of small-scale experiments.

In the present study, we optimized lactic acid production in SSF by *Lactobacillus amylovorus* NRRL B-4542 using Taguchi methodology. To the best of our knowledge, no reports are available on lactic acid production optimization by *Lactobacillus amylovorus* NRRL B-4542 in SSF using Taguchi Methodology.

The microorganism *Lactobacillus amylovorus* NRRL B-4542 used in this study was obtained from the USDA Northern Regional Research Center, Peoria, Illinois.

Lactobacillus amylovorus NRRL B-4542 was maintained on Cheng et al. (1991) media, (0.034 g $FeSO_4$, 1.0 g Sodium acetate 1.23 g $MgSO_4 \cdot 7H_2O$, 0.034 g $MnSO_4 \cdot H_2O$, 0.65 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g KH_2PO_4, 30 g yeast extract, 10 g sol-

uble starch, 15 g agar/l medium, pH 5.5). Slants were prepared with Cheng *et al.* Medium, inoculated and incubated at 40°C for 24 h, and stored at 4°C until further use.

The organism was grown in 50 ml Cheng *et al.* (1991) liquid medium in 250 ml conical flask containing 1% soluble starch by incubating at 40°C for 18 h, to obtain the required cell concentration (O.D. 1.7 at 660 nm). The above cells were centrifuged, washed, and used as inocula in Solid State Fermentation.

Total lactic acid was estimated according the method of Kimberley and Taylor (1996).

Taguchi has established OAs (Orthogonal Arrays) to describe a large number of experimental situations, mainly to reduce experimental errors and to enhance the efficiency and reproducibility of laboratory experiments. The symbolic designation of these arrays indicates the main information on the size of the experimentation, e.g.

Table 1. Factors and their levels assigned to different columns

Factor	Level 1	Level 2	Level 3
Temperature (°C)	35	40	--
pH	4.5	5.5	6.5
Inoculum (%, v/w)	14	17	20
moisture (%, v/w)	50	60	70
Yeast Extract (%, w/v)	1.0	2.0	3.0
$MgSO_4 \cdot 7H_2O$ (%, w/v)	0.05	0.1	0.15
Tween 80 (%, v/v)	0.05	0.1	0.15
CSL (%, w/v)	0.5	1.0	1.5

Table 2. L18 (2^1 X 3^7) Orthogonal Array

Experiment No.	Column								% of Lactic acid production (g/g Substrate)
	1	2	3	4	5	6	7	8	
1	1	1	1	1	1	1	1	1	83.4
2	1	1	2	2	2	2	2	2	83.5
3	1	1	3	3	3	3	3	3	88.9
4	1	2	1	1	2	2	3	3	87.5
5	1	2	2	2	3	3	1	1	88.5
6	1	2	3	3	1	1	2	2	84.5
7	1	3	1	2	1	3	2	3	89.7
8	1	3	2	3	2	1	3	1	82.5
9	1	3	3	1	3	2	1	2	86.7
10	2	1	1	3	3	2	2	1	89.8
11	2	1	2	1	1	3	3	2	90.9
12	2	1	3	2	2	1	1	3	83.4
13	2	2	1	2	3	1	3	2	91.5
14	2	2	2	3	1	2	1	3	85.3
15	2	2	3	1	2	3	2	1	84.3
16	2	3	1	3	2	3	1	2	88.5
17	2	3	2	1	3	1	2	3	87.4
18	2	3	3	2	1	2	3	1	89.9

L18 has 18 trials. The total number of degrees of freedom available in an OA is equal to the number of trials minus one. Each column consists of a number of conditions depending on the levels assigned to each factor. In the present study, all 8 columns are assigned with different factors, as indicated in Table 1. The temperature factor has been assigned with only two levels. Hence, it has nine level 1 and nine level 2 conditions (2^1). While, in the case of the other seven factors, each factor is assigned with three levels. Therefore, these factors have six level 1, six level 2, and six level 3 conditions (3^7). Table 2 shows the layout of the L18 $(2^1 \times 3^7)$ orthogonal array used in the present study.

All of the combination experiments using the assigned parameter values were conducted using a 250 ml conical flask containing 10 g of deoiled groundnut cake as a solid support and 10% raw corn starch as substrate, 0.034 g $FeSO_4$, 0.034 g $MnSO_4 \cdot H_2O$, 0.65 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g KH_2PO_4 / l medium were added and sterilized by autoclaving at 121°C for 15 min, dried, and kept ready for inoculations. For this, required concentrations of inoculum and other media components, previously weighed and sterilized, were added according to the DOE and were then incubated at temperature of 37°C and 40°C for 72 h. After 72 h of fermentation, the culture broth was separated and analyzed for lactic acid (Table 2).

QUALITEK 4 Software for automatic design and analysis of Taguchi experiments was used to study the following objectives of the analysis. Identification of the individual influence of each factor. Determination of the optimum condition. Estimation of performance at the optimum condition.

The main effects of the factors at the assigned levels on lactic acid production are depicted in Table 3. The difference between the average value of each factor at levels 2 and 1 indicates the relative influence of the effect; the larger the difference, the stronger the influence. The sign of the difference (+ or -) indicates whether the change from level 1 to level 2 increased or decreased the result (Table 3). Based on this data, it can be seen that yeast extract and inoculum showed strong negative influences

when switched from one level to another. Strong positive influence was noticed in terms of temperature and $MgSO_4 \cdot 7H_2O$ with the assigned levels (Table 3). However, when interactions of different factors were calculated (Table 4), it is interesting to note that the factors perceived to be the least significant, such as CSL, pH, and moisture (at their individual levels), showed the highest severity index percentages. Similarly, the severity index percentage for CSL (the weaker impact factor) vs $MgSO_4 \cdot 7H_2O$ (the stronger impact factor) was 71.01. These results suggest that the influence of one factor on lactic acid production was dependent upon the condition of the other factors in optimizing lactic acid production process parameters.

The percentage contributions of each factor are shown in an ANOVA Table 5. The last column of the ANOVA indicates the influence of each factor. Yeast extract was the most significant factor for lactic acid production. The confidence level for the yeast extract factor was observed to be 99.84%, while $MgSO_4 \cdot 7H_2O$ was significant with a confidence level of 99.31%. Moisture, pH, and CSL factors showed negligible influences on the lactic acid yield at their individual levels.

Optimum conditions and their performance in terms of contribution to achieving maximum yield of lactic acid production were given in Table 6. It can be seen from the table that factors such as yeast extract, $MgSO_4 \cdot 7H_2O$, and Tween 80 play more significant roles in product formation than do the other selected parameters and their levels. The expected result at optimum conditions was 95.80%, the actual yield at optimum conditions was 93.50%.

Pooling was done until the degrees of freedom (DOF) of the error term became close to half of the total of the experimental DOF. Thus the pH, moisture, and CSL were pooled. These ANOVA are shown in Table 7. Temperature, inoculum, yeast extract, $MgSO_4 \cdot 7H_2O$, and Tween 80 were significant to the overall process, with confidence levels of 98.98, 97.70, 99.84, 99.31, and 98.71, respectively.

After pooling of optimum conditions, performance was given in Table 8. The formation of product under optimal conditions was estimated using only the significant factors. Among all of the factors studied, yeast extract, $MgSO_4 \cdot 7H_2O$, Tween 80, and inoculum were most important for lactic acid production. These results indicate that the expected result under optimal conditions was 94.04%, while the actual yield at optimum conditions was 93.10%.

The pH, therefore, can be left at 5.5 (level 2) without adjustment with acid. The moisture can be held at 60% (level 2), and the CSL can be held at 0.5 g (level 1) for economical consideration. The levels of other factors should be held as indicated in Table 8.

A combination of factors and their levels involved in

Table 3. Main effects of the factors at the assigned levels on lactic acid production

Factor	Level 1	Level 2	Level 3	L2 - L1
Temperature (°C)	86.133	87.888	--	1.755
pH	86.649	86.933	87.450	0.284
Inoculum (%, v/w)	88.399	86.350	86.283	-2.050
moisture (%, v/w)	86.700	87.750	86.583	1.049
Yeast Extract (%, w/v)	87.283	84.950	88.799	-2.333
$MgSO_4 \cdot 7H_2O$ (%, w/v)	85.450	87.116	88.466	1.665
Tween 80 (%, v/v)	85.966	86.533	88.533	0.567
CSL (%, w/v)	86.400	87.599	87.033	1.198

Table 4. Estimated interaction of severity index for different parameters

Factors	Columns[@]	SI (%)[$]	Col*	Levels[&]
$MgSO_4 \cdot 7H_2O$ xCSL	6 x 8	71.01	14	[2,1]
moisture x CSL	4 x 8	62.14	12	[2,1]
Inoculum x moisture	3 x 4	61.94	7	[1,2]
pH x moisture	2 x 4	59.54	6	[2,2]
Inoculum x Tween80	3 x 7	49.04	4	[1,2]
pH x Tween 80	2 x 7	44.23	5	[1,3]
Inoculum x Yeast Extract	3 x 5	42.81	6	[1,3]
Temparature (°C) x pH	4 x 5	38.93	1	[2,3]
moisture x Yeast Extract	1 x 2	38.49	3	[2,3]
pH x $MgSO_4 \cdot 7H_2O$	2 x 6	37.30	4	[1,3]
Yeast extract x Tween 80	5 x 7	36.92	2	[1,3]
pH x Yeast Extract	2 x 5	35.87	7	[2,3]
Tween80 x CSL	7 x 8	32.63	15	[3,2]
pH x Inoculum	2 x 3	31.37	1	[2,1]
moisture x $MgSO_4 \cdot 7H_2O$	4 x 6	21.87	2	[2,3]
pH x CSL	2 x 8	20.83	10	[3,3]
Temparature (°C) x CSL	1 x 8	19.99	9	[2,2]
Temparature (°C) x Yeast Extract	1 x 5	19.07	4	[2,3]
Inoculum x CSL	3 x 8	18.88	11	[1,2]
Temparature (°C) x Tween80	1 x 7	17.21	6	[2,3]
Inoculum x $MgSO_4 \cdot 7H_2O$	3 x 6	15.21	5	[2,3]
$MgSO_4 \cdot 7H_2O$ x Tween80	6 x 7	14.61	1	[3,3]
Temparature (°C) x $MgSO_4 \cdot 7H_2O$	1 x 6	13.77	7	[1,3]
Yeast extract x CSL	5 x 8	13.47	13	[3,1]
Temparature (°C) x moisture	1 x 4	10.67	5	[2,2]
Yeast extract x $MgSO_4 \cdot 7H_2O$	5 x 6	7.48	3	[1,3]
moisture x Tween 80	4 x 7	1.32	3	[2,3]
Temparature (°C) x inoculum	1 x 3	0.32	2	[2,1]

[@]Columns - Represent the column locations to which the interacting factors are assigned.
[$]SI - interaction severity index (100% for 90 degree angle between the lines, 0% for parallel lines.
*Col - Shows column that should be reserved if this interaction effect were to be studied (2-L factors only).
[&]Opt - Indicates the factor levels desirable for the optimum conditions (based strictly on the first 2 levels). If an interaction is included in the study and found to be significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum conditions without consid erations of any interaction effects.

Table 5. Analysis of Variance (ANOVA)

Factors	DOF	Sums of squares	Variance	F-Ratio	Pure sum	percent
Temperature (°C)	1	13.870	13.870	277406.313	13.870	10.136
pH	2	1.963	0.981	19638.797	1.963	1.435
Inoculum (%, v/w)	2	17.371	8.685	173716.433	17.371	12.694
moisture (%, v/w)	2	4.959	2.479	49597.250	4.959	3.624
Yeast Extract (%, w/v)	2	45.131	22.565	451315.759	45.131	32.981
$MgSO_4.7H_2O$ (%, w/v)	2	27.406	13.703	274063.475	27.406	20.027
Tween 80 (%, v/v)	2	21.815	10.907	218151.096	21.815	15.941
CSL (%, w/v)	2	4.321	2.160	43217.669	4.321	3.158
Other/Error	2	-0.001	-0.001			0.004
Total	17	136.836				100.00%

the production of lactic acid by *Lactobacillus amylovorus* NRRL B-4542 was identified for obtaining maximum yield. The DOE using the Taguchi protocol has proved to be economical in optimization of lactic acid production.

From the analysis, it is evident that the yeast extract, $MgSO_4 \cdot 7H_2O$, and Tween 80 were found to be insignificant, while the rest of the factors were determined to be significant with confidence levels of 70% and above. The

Table 6. Optimal Conditions and Performance

Factors	Level Description.	Level	contribution
Temperature (°C)	40	2	0.877
pH	6.5	3	0.438
Inoculum (%, v/w)	14	1	1.388
moisture (%, v/w)	60	2	0.738
Yeast Extract (%, w/v)	3	3	1.788
MgSO$_4$.7H$_2$O (%, w/v)	0.15	3	1.455
Tween 80 (%, v/v)	0.15	3	1.522
CSL (%, w/v)	1	2	0.588
Total contribution from all factors			8.793
Current grand average performance			87.011
Expected result at optimal conditions			95.805

Table 7. Analysis of pooled Variance (POOLED ANOVA)

Factors	DOF	Sums of squares	Variance	F-Ratio	Pure sum	Percent
Temperature (°C)	1	13.87	13.87	9.867	12.464	9.108
pH	(2)	(1.963)		POOLED (CL = 98.84)		
Inoculum (%, v/w)	2	17.371	8.685	6.179	14.56	10.640
moisture (%, v/w)	(2)	(4.959)		POOLED (CL = 92.18%)		
Yeast Extract (%, w/v)	2	45.131	22.565	16.053	42.32	30.926
MgSO$_4$.7H$_2$O (%, w/v)	2	27.406	13.703	9.748	24.594	17.973
Tween 80 (%, v/v)	2	21.815	10.907	7.759	19.033	13.887
CSL (%, w/v)	(2)	(4.321)		POOLED (CL = 77.07)		
Other/Error	8	11.245	1.405			17.466
Total	17	136.84				100.00%

Table 8. Optimal Conditions and their Performance in Production of Lactic Acid

Factors	Level Description	Level	Contribution
Temperature (°C)	40	2	0.877
Inoculum (%, v/w)	14	1	1.388
Yeast Extract (%, v/w)	3	3	1.788
MgSO$_4$ · 7H$_2$O (%, v/w)	0.15	3	1.455
Tween 80 (%, v/w)	0.15	3	1.522
Total contribution from all factors			7.03
Current grand average performance			87.011
Expected result at optimal conditions			94.041

DOE using the Taguchi methodology for optimizing the yield of lactic acid production was an efficient strategy.

We are grateful to R.K.Roy, Michigan, USA for providing us with QUALITEK-4 software.

References

Abdullatif, T. and Y. Shang-Tian. 2002. Production of L(+)- lactic acid from glucose and starch by immobilized cells of Rhizopus oryzae in rotating fibrous bed bioreactor. Wiley Periodicals, Inc.

Ana, B.M., L.A. Jose, and C.P. Juan. 2001. Strategies to improve the bioconversion of processed wood into lactic acid by simultaneous saccharification and fermentation. *J. Chem. Technol. Biotechnol.* 76, 279-284

Cheng, P., R. Mueller, S. Jaeger, R. Bajpai, and G. Iannotti. 1991. Lactic acid production from enzyme thinned corn starch using *Lactobacillus amylovorus. J. Ind. Microbiol.* 7, 27-34.

Donilo, P., M.B. Michele, B. Luca, M. Rossella, B. Davide, C. Vittorio, L. Jefferson, L. Chi-li, M.R. Bianca, F. Laura, and A. Lilia. 1999. Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. *Appl. Eviron. Microbial.* 65. 4211-4215.

Hassan, K.S., B. Moldes, G.K. Richard, and J.S. Richard. 2001. Lactic acid production by simultaneous saccharification and fermentation of alfalfa fiber. *J. Biosc. Bioeng.* 92, 518-523.

Kimberley, A.C. and C. Taylor. 1996. A simple colorimetric assay for muramic acid and lactic acid. *Appl. Biochem. Biotechnol.* 56, 49-58.

Kotzamanidis, C.H., T. Roukas, and G. Skaracis. 2002. Optimization of lactic acid production from beet molasses by *Lactobacillus delbrueckii* NCIMB 8130. *World J. Microbiol. Biotech.* 18, 441-448.

Krishna, P.K. and S. Padma. 1998. Solid state cultivation of *Streotomyces clavuligerus* for production of Cephamycin C. *J. Scien. Indust. research.* 57, 587-590.

Litchfield, J.H. 1996. Microbiological production of lactic acid. *Advan. Appl. Microbiol.* 42, 45-95.

Pandey, A. 1992. Recent developments in solid state fermentation. *Process Biochem.* 27, 109-116.

Pandey, A., P. Selvakumar, P. Nigam, and C.R. Soccol. 1999. Solid state fermentation for production of industrial enzymes. *Curr Sci.* 77, 149-162.

Payot, T., Z. Chemaly, and M. Fick. 1999. Lactic acid production by *Bacillus coagulans* kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme. Microbial. Technology* 24, 191-199.

Ross, P.J. 1996. Taguchi techniques for quality engineering, McGraw Hill book company, New York.

Roy, R.K. 1990. A primer on the Taguchi Method. Society of Manufacturing Engineers, Dearborn, MI 48121, USA.

Roy, R.K. 2001. Design of Experiments Using the Taguchi Approach: 16 Steps to Product and Process Improvement. John Wiley & Sons, New York.

Soccol, C.R. and P.S. Luciana. 2003. Overview of applied solid-state fermentation in Brazil. *Biochem. Engin. Journal* 13, 205-218.

Sudip, R., D.G. Ravindra, K.V. Venkatesh, and S.S. Sunil, 2001. Optimal control strategies for simultaneous saccharification and fermentation of strach. *Process. Biochem.* 36, 713-722.

Taguchi, G. 1986. Introduction to quality engineering, quality resources, Asian Productivity Organization, NY.

Venkatesh, K.V. 1997. Simultaneous saccharification and fermentation of cellulose to lactic acid. *Bioresource Technology* 62, 91-98.

Xiaodong, W., G. Xuan, and S.K. Rakshit. 1997. Derect fermentative production of lactic acid on cassava and other starch substrates. Biotechnol. Letters, 19(9), 841-843.

Xuexia,Y., C.Hongzhang, G. Hongliang, and L. Zuohu. 2001. Bioconversion of corn straw by coupling ensiling and solid-state fermentation. *Bioresource. Technol.* 78, 277-280.