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Simultaneous Production of Nisin and Lactic Acid from Cheese Whey

Optimization of Fermentation Conditions Through Statistically Based Experimental Designs

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Abstract

A biorefinery process that utilizes cheese whey as substrate to simultaneously produce nisin, a natural food preservative, and lactic acid, a raw material for biopolymer production, was studied. The conditions for nisin biosynthesis and lactic acid coproduction by *Lactococcus lactis* subsp. *lactis* (ATCC 11454) in a whey-based medium were optimized using statistically based experimental designs. A Plackett-Burman design was applied to screen seven parameters for significant factors for the production of nisin and lactic acid. Nutrient supplements, including yeast extract, $MgSO_4$, and KH_2PO_4, were found to be the significant factors affecting nisin and lactic acid formation. As a follow-up, a central-composite design was applied to optimize these factors. Second-order polynomial models were developed to quantify the relationship between nisin and lactic acid production and the variables. The optimal values of these variables were also determined. Finally, a verification experiment was performed to confirm the optimal values that were predicted by the models. The experimented results agreed well with the model prediction, giving a similar production of 19.3 g/L of lactic acid and 92.9 mg/L of nisin.

Index Entries: Nisin; whey; fermentation; optimization; experimental design.

Introduction

Cheese whey is a byproduct of the dairy industry obtained by separating the coagulum from whole milk, cream, or skim milk. About 30 million t of liquid whey is produced annually in the United States alone.

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Cheese whey represents about 85–90% of the milk volume and retains 55% of the milk nutrients. Among the most abundant of these nutrients are lactose (4.5–5.0% [w/v]), soluble proteins (0.6–0.8% [w/v]), and mineral salts (0.5–0.7% [w/v]) *(1)*. Unfortunately, this byproduct and its associated nutritional quality have traditionally been treated as a waste because of its low value, low concentration, and limited market *(2)*. As a waste stream, though, cheese whey represents a major disposal and pollution problem because of its high biological oxygen demand (BOD) and chemical oxygen demand (COD) levels (BOD_5 = 30,000 – 50,000 and COD = 60,000 – 80,000) *(1)*. Thus, finding an environmentally friendly and economically advantageous method of disposal for whey is of great interest to the dairy industry, and using microbial cultures on cheese whey to produce value-added products is now considered the most profitable solution to the present disposal dilemma *(3)*.

Nisin is an antimicrobial peptide produced by certain *Lactococcus* species *(4)*. The peptide has strong antimicrobial activity against almost all Gram-positive bacteria and their spores, especially several food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and psychotrophic enterotoxigenic *Bacillus cereus (5–7)*. As a result of its antimicrobial properties, nisin has been accepted as a safe and natural preservative in more than 50 countries and is widely used in the food industry *(8)*. The Food and Drug Administration views nisin derived from *Lactococcus lactis* subsp. *lactis* to be a generally recognized as safe substance for use as an antimicrobial agent *(9)*, and, therefore, direct addition of nisin to various types of foods, such as cheese, margarine, flavored milk, and canned foods, is permitted *(10)*. In addition, nisin is also being considered for use in health and cosmetic products *(11)*.

In the current industrial process, nisin is manufactured by fermentation of *L. lactis* subsp. *lactis* in a milk-based medium. Biosynthesis of nisin is coupled with the growth of lactic acid bacteria and the production of a significant amount of lactic acid *(7)*. Lactic acid is an important chemical for food processing. It can also be used as a raw material in the production of the biodegradable polymer poly(lactic) acid *(12)*. Unfortunately, lactic acid is not recovered in the current nisin process.

Several studies *(13,14)* have indicated that cheese whey could also be used as feedstock for the production of nisin given supplementation of some essential nutrients. However, the systematic investigation of lactic acid formation accompanied by nisin biosynthesis, and the possibility of the simultaneous production of nisin and lactic acid, have not been reported.

In the present work, the major variables that have significant effects on nisin biosynthesis and lactic acid coproduction from whey were identified, and the optimal conditions for the production of nisin and lactic acid were determined respectively using statistically based experimental designs. In this article, we also discussed the feasibility of simultaneous production of nisin and lactic acid from cheese whey.

Materials and Methods

Microorganisms, Media, and Cultivation

L. lactis subsp. *lactis* (ATCC 11454) was the nisin-producing microorganism used in this work. *Micrococcus luteus* (ATCC 9341) was used as an indicating microorganism in the bioassay of nisin concentrations. The compositions of media used for the growth of microorganisms are summarized as follows. Medium I, used for seed culture of *L. lactis* (pH 7.0), contained 5 g/L of glucose, 5 g/L of polypeptone, and 5 g/L of yeast extract. Medium II, used for bioassay of nisin (pH 7.0), contained 10 g/L of glucose, 5 g/L of polypeptone, 5 g/L of yeast extract, and 5 g/L of NaCl. Medium III, used for the main fermentation, contained 50 g/L of sweet whey powder (provided by WesternFarm Food, Seattle, WA) and a predetermined amount of other nutrients shown in the experimental designs. $CaCO_3$ powder (30 g/L) was added in medium III in order to maintain a stable pH during fermentation.

Seed culture of *L. lactis* was conducted in 125-mL Erlenmeyer flasks placed on an orbital shaker at 160 rpm and 30°C for 8 h. Main fermentations were performed in 250-mL Erlenmeyer flasks containing 100 mL of medium III and 5 mL of the seed medium.

Analysis

Nisin concentration was measured using a bioassay procedure based on the method of Shimizu et al. *(15)*. A high-performance anion-exchange chromatography method *(16)* was used for lactic acid analysis.

Plackett-Burman Experimental Design

The Plackett-Burman (PB) design has proven very effective and has been widely used to identify significant variables from a larger number of potential variables (>5) with a minimum of testing *(17,18)*. A 12-run PB design was used to identify which variables have significant effects on nisin and lactic acid production by *L. lactis*. The design matrix shown in Table 1 was developed according to Greasham and Herber *(17)*. Seven variables (A–G)—i.e., yeast extract, polypeptone, KH_2PO_4, $MgSO_4$, surfactant (Tween-80), pH, and temperature—were chosen as the candidate factors based on Parente and Ricciardi's review *(7)*. D_1 to D_4 in Table 1 are dummy factors employed to evaluate the standard errors of the experiment. Low levels (–1) and high levels (+1) were assigned for each factor. Two flasks in parallel were performed for each condition. The average value of nisin and lactic acid concentrations after 24 h of fermentation were used as the responses in this design.

Greasham and Herber *(17)* described in detail the statistical analyses used to identify the significance of the variables. The significance of variables was determined by student's *t*-test. Variables with p values < 0.2 were considered significant.

Table 1
PB Design of Variables with Nisin and Lactic Acid Concentration as Response

	Variable											Response	
Run	A	B	C	D	E	F	G	D_1	D_2	D_3	D_4	Nisin (mg/L)	Lactic acid (g/L)
1	+1	+1	–1	+1	+1	+1	–1	–1	–1	+1	–1	80.2	19.0
2	–1	+1	+1	–1	+1	+1	+1	–1	–1	–1	+1	14.6	7.8
3	+1	–1	+1	+1	–1	+1	+1	+1	–1	–1	–1	87.7	19.1
4	–1	+1	–1	+1	+1	–1	+1	+1	+1	–1	–1	85.9	18.1
5	–1	–1	+1	–1	+1	+1	–1	+1	+1	+1	–1	11.5	7.9
6	–1	–1	–1	+1	–1	+1	+1	–1	+1	+1	+1	88.2	18.3
7	+1	–1	–1	–1	+1	–1	+1	+1	–1	+1	+1	11.2	6.7
8	+1	+1	–1	–1	–1	+1	–1	+1	+1	–1	+1	10.7	7.3
9	+1	+1	+1	–1	–1	–1	+1	–1	+1	+1	–1	13.1	6.4
10	–1	+1	+1	+1	–1	–1	–1	+1	–1	+1	+1	79.0	11.1
11	+1	–1	+1	+1	+1	–1	–1	–1	+1	–1	+1	84.3	17.8
12	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	6.4	6.1

Table 2
Central Composite Design of Factors in Coded Levels with Nisin and Lactic Acid Concentration as Response

Run	Type	*A*	*B*	*C*	Nisin (mg/L)	Lactic acid (g/L)
1	Center	0	0	0	87.7	18.8
2	Center	0	0	0	87.0	19.0
3	Center	0	0	0	87.4	18.8
4	Center	0	0	0	87.1	18.9
5	Center	0	0	0	87.5	18.9
6	Center	0	0	0	86.8	18.9
7	Axial	0	0	–1.68	78.3	19.0
8	Axial	0	0	1.68	86.8	19.0
9	Axial	–1.68	0	0	63.5	17.3
10	Axial	0	–1.68	0	80.6	18.2
11	Axial	0	1.68	0	87.6	19.1
12	Axial	1.68	0	0	89.0	19.1
13	Fact	1	–1	1	88.1	18.6
14	Fact	1	1	–1	88.0	18.8
15	Fact	–1	1	1	64.9	17.5
16	Fact	–1	–1	–1	60.9	16.9
17	Fact	–1	1	–1	63.4	17.5
18	Fact	1	1	1	88.9	19.1
19	Fact	–1	–1	1	62.1	16.9
20	Fact	1	–1	–1	85.1	18.2

Table 3
Coded and Actual Values of Factors in Central Composite Design

Factor	Name	Units	Axial (–1.68)	Low (–1)	Central (0)	High (+1)	Axial (+1.68)
A	Yeast extract	g/L	6.64	8.00	10.00	12.00	13.36
B	KH_2PO_4	g/L	0.08	0.25	0.50	0.75	0.92
C	$MgSO_4$	g/L	0.08	0.25	0.50	0.75	0.92

Central Composite Experimental Design

Once the variables having the greatest influence on the responses were identified, a 20-run central composite design was used to optimize the levels of these variables *(18)*. A design matrix was developed (Table 2) and the true values for the variables were determined (Table 3).

After the responses were obtained, they were subjected to multiple nonlinear regression and optimization using the software Design-Expert (V6.0; Stat-Ease, Minneapolis, MN). Second-order polynomial models were applied to correlate these variables. Only the estimates of coefficients with significant levels higher than 90% (i.e., $p < 0.10$) were included in the final model. An *F*-test was used to evaluate the significance of the models.

Table 4
Variables Screened in PB Design and Their Real Values

	Variable						
	A (pH)	B (Temperature) (°C)	C (Tween-80) (g/L)	D (Yeast extract) (g/L)	E (Polypeptone) (g/L)	F (KH_2PO_4) (g/L)	G ($MgSO_4$) (g/L)
Low level (–1)	5.5	30	0	0	0	0	0
High level (+1)	6.5	37	1	1	10	1	1

Table 5
Effects of Variables in PB Design on Nisin and Lactic Acid Production and Associated Statistical Tests[a]

	Nisin			Lactic acid		
Variables	Effects (mg/L)	*t* value	*p* value	Effects (g/L)	*t* value	*p* value
pH	1.6	0.20	0.85	7.0	1.08	0.34
Temperature	–5.8	–0.71	0.52	–6.2	–0.76	0.49
Tween-80	7.6	0.93	0.41	–5.4	–0.66	0.55
Yeast extract	437.8	53.78	0.00	61.2	7.49	0.00
Polypeptone	2.6	0.32	0.77	9.0	1.10	0.33
KH_2PO_4	13.0	1.60	0.18	13.2	1.62	0.18
$MgSO_4$	28.6	3.51	0.02	7.2	0.88	0.43

[a] Significant values are underlined.

Results and Discussion

Screening Experiment

Table 4 gives the seven variables chosen as candidate factors with their assigned low and high levels. Table 1 provides the 12-run PB design used to identify which variables have significant effects on nisin and lactic acid production by *L. lactis*. Table 5 shows the resulting effects of the variables on the responses and the associated *t*-values and significant levels.

The results showed that yeast extract, KH_2PO_4, and $MgSO_4$, in the tested range, had significant effects on nisin biosynthesis while the significant factors for lactic acid coproduction were yeast extract and KH_2PO_4. The results in Table 5 clearly show that nisin biosynthesis and lactic acid production were dramatically influenced by the composition of the medium. This is because the nisin-producing strain *L. lactis* is a well-known nutritionally fastidious microorganism *(19)*, and, therefore, an abundance of nutrients is required for cell growth and metabolism.

De Vuyst *(20)* found that the amino acids serine, threonine, and cysteine highly stimulated nisin production, indicating their precursor role during nisin biosynthesis. In the present work, the significant effect of yeast extract in the tested concentration range supported the De Vuyst's findings. The significant effects of inorganic phosphate and magnesium sulfate on nisin biosynthesis revealed in our work are also consistent with the results of De Vuyst and Vandamme *(21)* and Meghrous et al. *(22)*.

The significance of yeast extract and KH_2PO_4 for lactic acid formation indicates the important role of protein and inorganic phosphate in the metabolism of *L. lactis*. The lesser influence of polypeptone compared with yeast extract proves that yeast extract is a good nitrogen source for nisin and lactic acid production. The influences of pH and temperature were not significant in this screening experiment, because this test was carried out close to the optimal conditions of these two variables *(16)*.

Optimization Using Surface Response Methodology

In the second optimization step, the exact values of the three variables that were identified to have significant effects on nisin and/or lactic acid production were determined using a central composite design (Table 2). The coded and actual values of each variable are given in Table 3. The fermentation media (pH 6.5) were composed of 50 g/L of whey, 5 g/L of polypeptone, 1 g/L of Tween-80, and 30 g/L of $CaCO_3$, and the predetermined amount of the three variables was assigned by the central composite design. The content of nisin and lactic acid after 24 h of fermentation at 30°C was measured and are presented as responses in Table 2.

The responses of nisin and lactic acid were correlated by nonlinear regression using the following full quadratic polynomial model:

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 \quad (1)$$

Table 6
Estimate of Coefficient of Factors and Associated Significant Levels[a]

	Nisin		Lactic acid	
Factor	Coefficient estimate	*p* value	Coefficient estimate	*p* value
Intercept	87.28	0.00	18.90	0.00
A	10.38	0.00	0.66	0.00
B	1.52	0.18	0.27	0.00
C	1.52	0.18	0.05	0.45
A^2	–5.07	0.00	–0.39	0.00
B^2	–2.30	0.05	–0.24	0.01
C^2	–2.86	0.02	–0.11	0.13
AB	–0.21	0.88	0.00	1.00
BC	–0.23	0.91	0.00	0.98
AC	0.16	0.87	0.07	0.43

[a]Significant values are underlined.

in which *Y* is the desired response; b_i are the coefficients; and *A*, *B*, and *C* are the three factors. Table 6 provides the results of coefficient estimates and the associated significance levels obtained from the Design-Expert software. The estimates with significance levels higher than 90% ($p < 0.10$) were included in the final model. The models that describe the responses of nisin and lactic acid as functions of the most significant variables are as follows:

$$\text{Nisin}\,(\text{mg/L}) = 87.28 + 10.38 \times [\text{Yeast extract}] - 5.07 \times [\text{Yeast extract}]^2 - 2.30 \times [\text{KH}_2\text{PO}_4]^2 - 2.86 \times [\text{MgSO}_4]^2 \quad (2)$$

$$\text{Lactic acid}\,(\text{g/L}) = 18.90 + 0.66 \times [\text{Yeast extract}] + 0.27 \times [\text{KH}_2\text{PO}_4] - 0.39 \times [\text{Yeast extract}]^2 - 0.24 \times [\text{KH}_2\text{PO}_4]^2 \quad (3)$$

in which [Yeast extract], [KH_2PO_4], and [$MgSO_4$] represent the coded values of yeast extract, KH_2PO_4, and $MgSO_4$, respectively. *F*-test showed that the models were reliable because the significance levels were >99%.

The three-dimensional surface plots presented in Figs. 1 to 4 demonstrate the effects of yeast extract, KH_2PO_4, and $MgSO_4$ on nisin and lactic acid production. The optimal value of each factor is also clearly shown in the plots. Figure 1 shows the function of yeast extract and KH_2PO_4 on nisin production when $MgSO_4$ was kept at the central point. Nisin concentration reached a maximum level with about 12 g/L of yeast extract and 0.6 g/L of KH_2PO_4. Figure 2 shows nisin biosynthesis as the function of yeast extract and $MgSO_4$ (KH_2PO_4 was kept at the central point). The optimal value of $MgSO_4$ for nisin biosynthesis was close to the central point. Figures 3 and 4 also show that the optimal conditions of lactic acid production were about 12 g/L of yeast extract, 0.6 g/L of KH_2PO_4, and 0.6 g/L of $MgSO_4$.

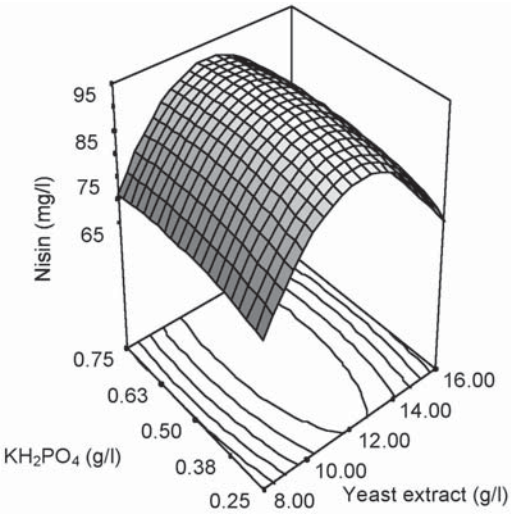

Fig. 1. Three-dimensional surface plot of nisin production as function of yeast extract and KH_2PO_4 ($MgSO_4$ was kept at central point).

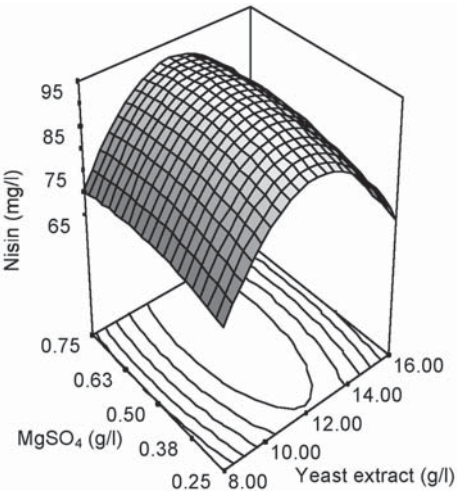

Fig. 2. Three-dimensional surface plot of nisin production as function of yeast extract and $MgSO_4$ (KH_2PO_4 was kept at central point).

The optimal conditions for nisin biosynthesis and lactic acid formation were obtained by further numerical analysis of the response surface using Design-Expert software and are presented in Table 7. The solution to the maximal nisin biosynthesis was 12.04 g/L for yeast extract, 0.57 g/L for KH_2PO_4, and 0.57 g/L for $MgSO_4$. The solution to the maximal lactic acid production was 11.78 g/L for yeast extract, 0.64 g/L for KH_2PO_4, and 0.63

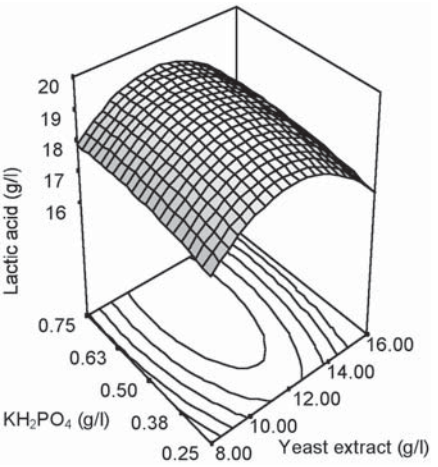

Fig. 3. Three-dimensional surface plot of lactic acid production as function of yeast extract and KH_2PO_4 ($MgSO_4$ was kept at central point).

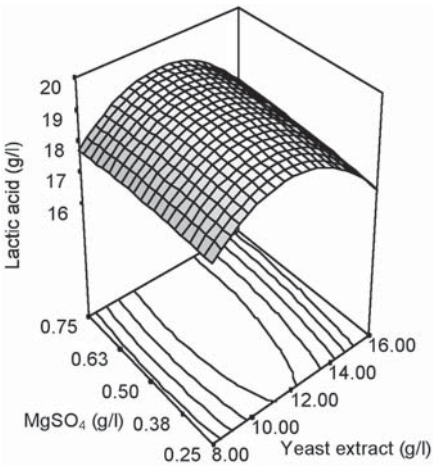

Fig. 4. Three-dimensional surface plot of nisin production as function of yeast extract and $MgSO_4$ (KH_2PO_4 was kept at central point).

g/L for $MgSO_4$. Finally, the solution to the simultaneous maximal production of nisin and lactic acid was 11.89 g/L for yeast extract, 0.61 g/L for KH_2PO_4, and 0.59 g/L for $MgSO_4$. The data in Table 7 also reveal that the predicated values of nisin and lactic acid under these three conditions have no significant difference. Thus, simultaneous production of nisin and lactic acid is feasible, since the optimal conditions for nisin biosynthesis and lactic acid formation by *L. lactis* using whey as feedstock are almost the same.

Table 7
Optimal Conditions for Nisin and Lactic Acid Production from Whey Obtained from Response Surface Analyse

Criteria		Optimal values			Predicated results	
Nisin	Lactic acid	Yeast extract	KH_2PO_4	$MgSO_4$	Nisin	Lactic acid
—	Maximum	11.78	0.64	0.63	92.5	19.3
Maximum	—	12.04	0.57	0.57	93.0	19.2
Maximum	Maximum	11.89	0.61	0.59	92.9	19.3

Verification

A verification experiment under the conditions of 12 g/L for yeast extract, 0.6 g/L for KH_2PO_4, and 0.6 g/L for $MgSO_4$ was conducted separately in order to confirm the optimal conditions obtained from the statistically based experimental designs. After 24 h of fermentation, 92.1 mg/L of nisin and 19.3 g/L of lactic acid were obtained. This result was very close to the predicted value of 92.9 mg/L of nisin and 19.3 g/L of lactic acid. In addition, the nisin result also agreed well with the "ceiling concentration" of nisin previously reported by Kim et al. *(23)*. Therefore, the optimal conditions predicted from the statistically based experimental designs were valid.

Conclusion

We aimed at optimizing lactic acid and nisin coproduction by *L. lactis* from cheese whey using statistically based experimental designs. The significant factors for nisin and lactic acid formation were screened and then quantitatively optimized using only 32 shake-flask trials. Compared with the conventional and time-consuming one-variable-at-a-time approach, the statistically based experimental designs proved to be efficient tools in this optimization process. However, considering industrial application, this work is only the very first step needed for bioprocess development. In particular, 19.3 g/L of lactic acid is too low for practical application, with this value of lactic acid not even able to compensate for separation costs. Therefore, further research on the improvement of nisin and lactic acid production and the associated development of effective product recovery methods is required.

Acknowledgments

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