0273-2289/1559–0291 (Online)/07/136–140/875–884/$30.00

Utilization of Condensed Distillers Solubles as Nutrient Supplement for Production of Nisin and Lactic Acid from Whey

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Abstract

The major challenge associated with the rapid growth of the ethanol industry is the usage of the coproducts, i.e., condensed distillers solubles (CDS) and distillers dried grains, which are currently sold as animal feed supplements. As the growth of the livestock industries remains flat, alternative usage of these coproducts is urgently needed. CDS is obtained after the removal of ethanol by distillation from the yeast fermentation of a grain or a grain mixture by condensing the thin stillage fraction to semisolid. In this work, CDS was first characterized and yeast biomass was proven to be the major component of CDS. CDS contained 7.50% crude protein but with only 42% of that protein being water soluble. Then, CDS was applied as a nutrient supplement for simultaneous production of nisin and lactic acid by *Lactococcus lactis* subsp. *lactis* (ATCC 11454). Although CDS was able to support bacteria growth and nisin production, a strong inhibition was observed when CDS was overdosed. This may be caused by the existence of the major ethanol fermentation byproducts, especially lactate and acetate, in CDS. In the final step, the CDS based medium composition for nisin and lactic acid production was optimized using response surface methodology.

Index Entries: Condensed distillers solubles; nutrient supplement; fermentation; lactic acid; *Lactococcus lactis*; nisin.

Introduction

Total ethanol production in United States has grown significantly in recent years. In year 2005, 95 ethanol plants in 19 states produced a record 3.904 billion gal of ethanol, an increase of 17% from 2004 and 126% from 2001. Dry mill ethanol refineries accounted for 79% of production capacity, and wet mills 21% *(1)*.The accelerated growth of ethanol industry is believed to be capable of reenergizing rural and farm development through increased

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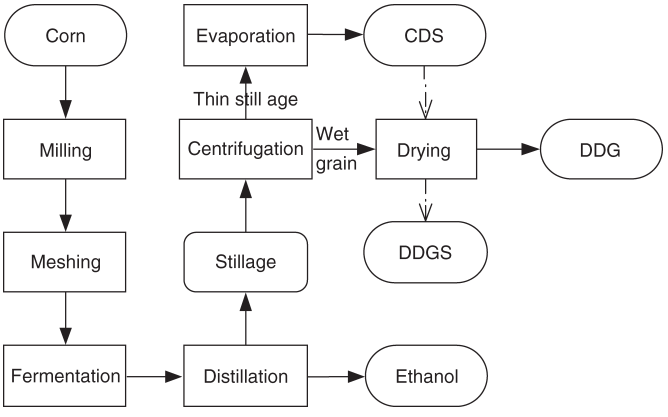

Fig. 1. Diagram of corn dry-milling process for ethanol and related coproducts production.

employment, enhancing local industry, and aiding our national security through increased reliance on domestic renewable energy and decreasing greenhouse gas emissions *(2)*.

The major challenge associated with the rapid development of ethanol industry is the usage of the coproducts, i.e., distillers dried grains (DDG) and condensed distillers solubles (CDS) *(3,4)*. In the dry mill ethanol process, as shown in Fig. 1 *(3,4)*, the corn kernels are first ground into a flour, or "meal," and mixed with water to form a slurry, called "mash." Enzymes are added to break down the starch to fermentable sugars. The mash is then pumped to the fermentors wherein yeast converts the sugars in the mash into ethanol. The fermented mash is pumped to the distillation tower wherein the ethanol is separated from the nonfermentable solids (the stillage). The stillage from the distillation system is sent through a centrifuge that separates the coarse grains from the solubles. The coarse grains are dried to produce DDG. Another coproduct, CDS, is also obtained in significant amount after the removal of ethanol by condensing the thin stillage fraction to a semisolid. In some dry-mill plants, CDS is mixed with the coarse grains from the centrifuge and then dried to produce dried distillers grains with solubles.

DDG and CDS are currently used as animal feed supplements *(3,4)*. As the growth of the livestock industries remain flat, dry mills will soon be faced with the need to identify new, potential customers to ensure that there are markets that will utilize these coproducts from the increasing number of dry mills coming online to meet the increasing ethanol demand. Therefore, alternative usage of CDS and DDG are urgently needed. The objective of this work is to study the feasibility of using CDS as a nutrient supplement for nisin and lactic acid coproduction from cheese whey. Nisin is an antimicrobial peptide produced by certain *Lactococcus* bacteria *(5)*, which has been accepted as a safe and natural preservative in more than 50 countries and is widely used as an antimicrobial agent in the food industry *(5–9)*. Biosynthesis of nisin is coupled with the growth of lactic acid bacteria and

lactic acid production *(8)*. 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 *(10)*. Our former study *(11)* has proved that simultaneous production of nisin and lactic acid is feasible, because the optimal conditions for nisin biosynthesis and lactic acid formation by *L. lactis* using whey as feedstock are almost the same. In this research, CDS was characterized and applied as nutrient supplement for simultaneous production of nisin and lactic acid from whey. Effects of CDS on nisin biosynthesis, lactic acid production, and bacteria growth were studied.

Materials and Methods

Characterization of CDS

The CDS used in this research was kindly provided by National Corn Growers Association, Chesterfield, Missouri. Analysis of total solids, total phosphorus, ammonia, total nitrogen, and crude protein in CDS were carried using Association of Official Analytical Chemists standard methods *(12)*. Water soluble peptides and amino acids were analyzed following the Ohnishi and Barr modified Lowry procedure *(13)* using the kit TP 0200 purchased from Sigma-Aldrich (St. Louis, MO). Acetate and lactate content in CDS were quantified by high-performance anion exchange chromatography using a Dionex DX-550 system. The detailed IC analysis conditions can be found at Liu et al. *(14)*.

Determination of the Major Component of CDS

A size distribution study was conducted according to the flowchart shown in Fig. 2. Hundred grams of CDS was thoroughly mixed with 900 mL water at which point the mixture was sieved using two standard sieves (produced by CSC Scientific Co., Inc., Fairfax, VA): no. 45 with an opening diameter of 0.355 mm and no. 120 with an opening diameter of 0.125 mm. The solid particles retained by each sieve were thoroughly washed using deionized water at room temperature, then dried at 105°C for 6 h and weighed.

Microorganisms and Media

Lactococcus lactis subsp. *lactis* (American Type Culture Collection [ATCC] 11454) was the nisin-producing microorganism used in this work. *Micrococcus luteus* (ATCC 9341) was used as an indicating microorganism in the bioassay used to measure nisin concentrations. The compositions of media used for the growth of these microorganisms are summarized as follows. Medium I, used for seed culture of *L. lactis* (pH 7.0), contained 5.0 g/L of glucose, 5.0 g/L of polypeptone, and 5.0 g/L of yeast extract (YE). Medium II, used for bioassay of nisin (pH 7.0), contained 10.0 g/L of glucose, 5.0 g/L of polypeptone, 5.9 g/L of YE, and 5.0 g/L of NaCl. Medium III, used for the main fermentation, contained 20.0 g/L of sweet whey powder (provided by WesternFarm Food Inc., Seattle, WA), 0.6 g/L for

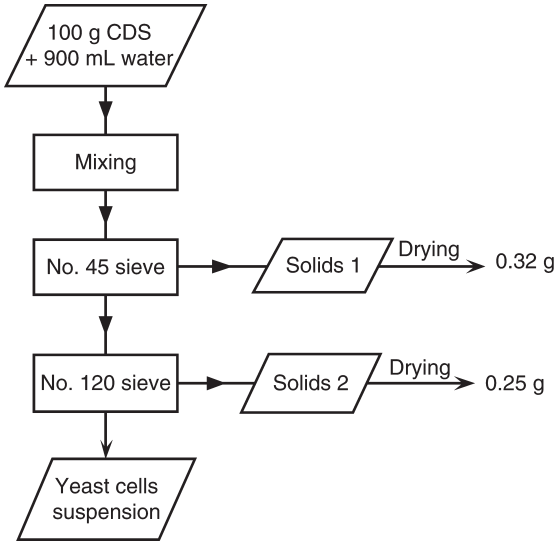

Fig. 2. Flowchart of CDS sieving experiment.

KH_2PO_4, 0.6 g/L for $MgSO_4$, and other nutrients, the amount of which are indicated in the experimental designs.

Cultivation Method

Before main cultivation was performed, culture size was scaled-up by two steps in order to increase the amount of cells with high growth activity. 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 a 5-L Bioflo 110 fermentor (New Brunswick Scientific, Edison, NJ) equipped with temperature, pH, dissolved oxygen concentration, and gas flow control systems. The working volume was 2 L. Air was supplied to the fermentor for aerobic cultivation conditions.

Analysis of Fermentation Products

The viable cell concentration of *L. lactis* was determined as colony-forming units on agar plates. Concentrations of L-lactic acid and acetic acid in the medium and fermentation broth were analyzed using the high-performance anion-exchange chromatography method mentioned under "CDS Characterization" *(14)*. Nisin concentration was measured by a bioassay method based on the method of Shimizu et al. *(15)*.

Results and Discussion

Characterization of CDS

Because the detailed information of CDS composition was not available, the corn dry-mill byproduct needed to be characterized before using

Table 1
Characterization of the Corn CDS Applied in This Research

Parameter	Content (g/100.0 g of CDS)
Total solids	28.70
Water	71.30
Total nitrogen	1.20
Total phosphorous	0.31
Crude protein	7.50
Water soluble peptides and amino acids	3.16
Ammonia	0.11
Lactate	2.40
Acetate	0.15

it as nutrient supplement for nisin fermentation. Total solids, total phosphorus, ammonia, total nitrogen, crude protein, and water soluble peptides and amino acid profiles of CDS were analyzed, respectively, using standard methods. The content of the major ethanol fermentation byproducts, including lactate and acetate, which may have negative effects on bacteria metabolism, were also measured. The results are shown in Table 1.

Yeast Biomass is the Major Component of CDS

CDS is the condensed thin stillage fraction of yeast fermentation broth left after the removal of ethanol by distillation. Therefore, CDS is a mixture of yeast cells and small grain particles. In order to know the major component in CDS, a sieve separation study was conducted. As shown in Fig. 2, a mixture of 100.0 g CDS and 900 mL water was sieved using two standard sieves, no. 45 with the opening diameter of 0.355 mm and no. 120 with the opening diameter of 0.125 mm. The solid particles retained by each sieve were thoroughly washed and dried up. Only 0.32 g of solids were retained by no. 45 sieve and 0.25 g of solids by no. 120 sieve. Compared with the total amount of CDS loaded in this experiment, 100.0 g wet base, which corresponds to 27.5 g dry weight, the percentage of the grain particles with the size larger than 0.125 mm in CDS was only 2.1%. Therefore, we can safely draw the conclusion that yeast biomass is the major component of CDS.

Water Soluble Nitrogen in CDS

As yeast biomass is the major component of CDS, a certain amount of organic nitrogen in yeast cells should have been leached out during the long-duration heat treatment of distillation. The results in Table 1 indicate that, CDS has 7.50% crude protein but with only 42% of that protein being water soluble. The content of ammonia in CDS is very low at 0.11% and only contributes to 8.6% of the total nitrogen.

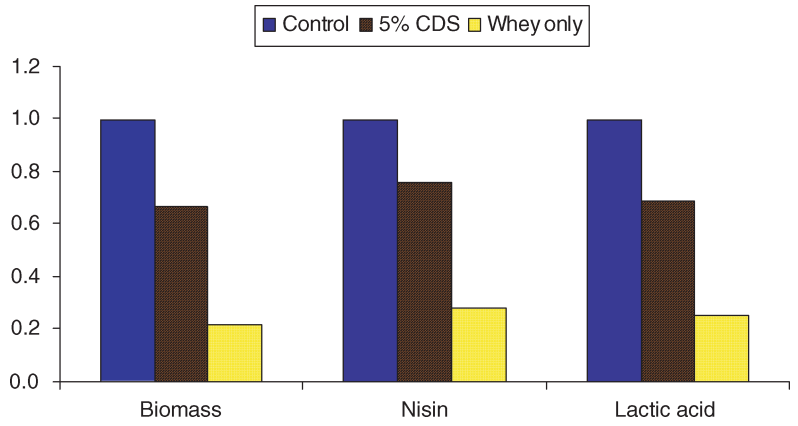

Fig. 3. Relative production of nisin, lactic acid, and biomass when 5% CDS alone was used as nutrient supplement.

CDS Alone as the Nutrient Supplement

In order to quantify the performance of CDS, the optimal medium for nisin and lactic acid coproduction obtained in an earlier study *(11)* was used as a positive control, and the whey without any nutrient added was used as a negative control. $CaCO_3$ was provided to buffer the fermentation pH around 5.5. The biomass, nisin biosynthesis, and lactic acid formation after 24 h of fermentation were compared. As shown in Fig. 3, the production of nisin was very poor when bacteria grew on whey without addition of any nutrient. A significant increase (twofold) of nisin formation was seen when 5% of CDS (wet base) was added, although it was only 70% of that at the earlier optimized conditions *(11)*. Therefore, it can be concluded that CDS does provide essential nutrients for nisin production from whey.

Inhibition of Nisin Biosynthesis Owing to CDS Overdose

As shown in Fig. 3, the stimulation of CDS on nisin production was observed when 5% of CDS (wet base) was added into fermentation medium. In the following experiment, more CDS was added into media in order to provide essential nutrients for nisin production from whey. Similarly, the optimal medium for nisin and lactic acid coproduction obtained in an earlier study *(11)* was used as a positive control, and the whey without any nutrient added was used as a negative control for the quantification of the performance of CDS. $CaCO_3$ was provided to maintain the fermentation pH around 5.5. The biomass, nisin biosynthesis, and lactic acid formation after 24 h of fermentation were compared. As shown in Fig. 4, the production of nisin decreased with the addition of more CDS in the media. The only explanation to this phenomenon is that CDS contains some inhibitory byproducts that have negative effects on the growth of lactic acid bacteria and the production of nisin. The byproducts of ethanol fermentation, i.e., acetate, lactate, and many others might be responsible for the inhibition observed.

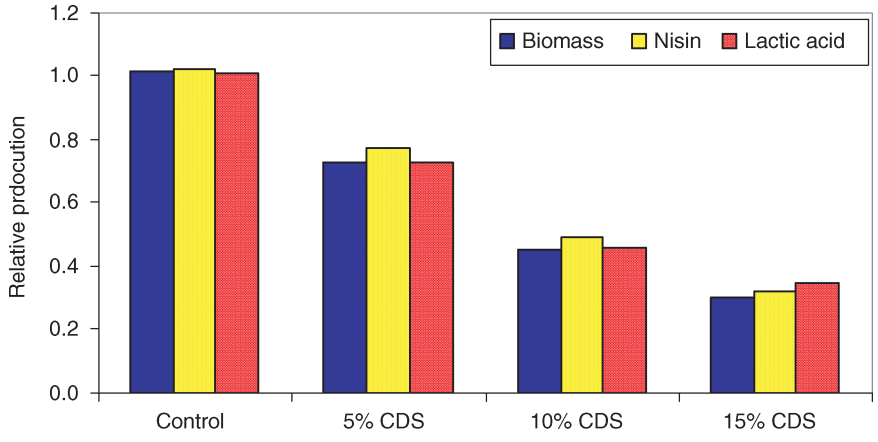

Fig. 4. Inhibition of biomass, nisin, and lactic acid production when CDS is overdosed.

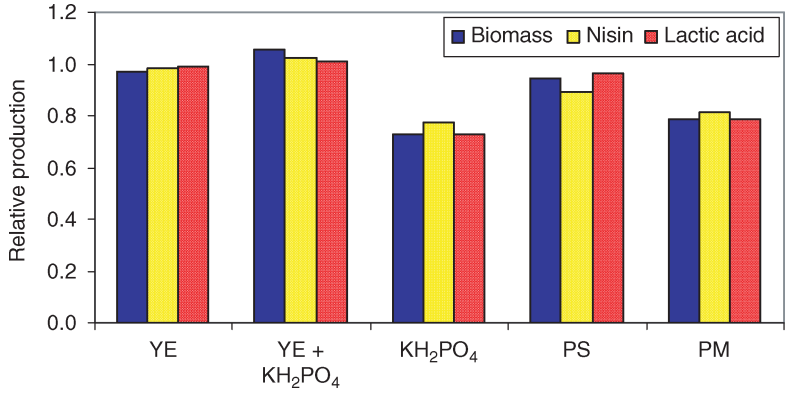

Fig. 5. Stimulation effects of different nutrient supplements on nisin, lactic acid, and biomass production (5% CDS + other nutrients). YE, yeast extract; PS, peptone from soy; and PM, peptone from meat.

Effects of Nutrient Supplements on Nisin and Lactic Acid Production

Considering that the nisin producing strain *L. lactis* is a well-known, nutritionally fastidious microorganism requiring an abundance of nutrients for cell growth and metabolism *(5)*, nutrients in addition to CDS may be required for the simultaneous production of nisin and lactic acid. YE, peptone from meat (PM), and peptone from soy (PS), the most widely used organic nutrient supplements in such fermentation studies, as well as KH_2PO_4, were selected as the candidates for nutrient supplementation. The stimulation effects of these nutrient supplements on cell growth, nisin formation, and lactic acid production were studied. In addition to 5% of CDS; YE, PS, PM, and KH_2PO_4 were added to the media. The fermentation results are compared in Fig. 5. Similarly in Fig. 3, the optimal medium for nisin and lactic acid coproduction obtained in an earlier study *(11)* was used as a positive control, and the whey without any nutrient added was

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

Run	Type	CDS	YE	Nisin (mg/L)	Biomass (10^8 colony forming units/mL)	Lactic acid (g/L)
1	Center	0	0	82	12.2	18.7
2	Center	0	0	81	12.6	18.7
3	Center	0	0	81	12.2	18.8
4	Center	0	0	80	12.6	18.9
5	Center	0	0	80	12.4	18.8
6	Axial	−1.41	0	67	8.4	11.8
7	Axial	+1.41	0	30	5.4	8.5
8	Axial	0	−1.41	56	8.4	13.6
9	Axial	0	+1.41	80	12.6	17.6
10	Fact	−1	−1	30	5	8.8
11	Fact	+1	−1	37	5.8	8.9
12	Fact	−1	+1	67	11.2	18.7
13	Fact	+1	+1	39	8	9.0

used as a negative control. YE and peptone, as seen by the comparative yields with the controls shown in Fig. 3, are ideal sources of nutrient for nisin and lactic acid coproduction. KH_2PO_4 is essential, as the yield of nisin and lactic acid was around 2.7 times of the negative control. The combination of KH_2PO_4 and YE gave the highest yield of nisin and lactic acid.

Medium Optimization Using Response Surface Methodology

The results shown in Figs. 3–5 indicate that, CDS is a good source of nutrients essential for nisin and lactic acid cofermentation; however, only a limited amount of CDS can be added for this purpose. Considering that CDS alone cannot provide sufficient nutrients for the well-known, nutritionally fastidious microorganism *L. lactis*, YE was selected as the auxiliary nutrient supplement in order to satisfy the requirement of nisin and lactic acid fermentation. The contents of them in the fermentation media were optimized through a statistically based design of experimentation in central composite design (Table 2) *(11)*. The coded and actual values of each variable are listed in Table 3. The fermentation media were made up of 50.0 g/L of whey, 1.0 g/L of KH_2PO_4, 30.0 g/L of $CaCO_3$, and the predetermined amount of the three variables were assigned by the central composite design. The content of nisin and lactic acid after 24 h of fermentation at 30°C were measured and presented as responses in Table 2.

After the responses were obtained, they were subjected to multiple nonlinear regression and optimization using the software Design-Expert (V6.0, 2001, Stat-Ease Inc., MN). The optimal conditions for nisin

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

Factor	Name	Axial (–1.41)	Low (–1)	Central (0)	High (+1)	Axial (+1.41)
A	CDS (g/L)	0.50	15.00	50.00	85.00	99.50
B	YE (g/L)	0.05	1.50	5.00	8.50	9.95

biosynthesis and lactic acid formation were obtained by further numerical analysis of the three-dimensional response surface plots using the software. The solution to the maximal nisin biosynthesis was 37.31 g/L for CDS and 7.12 g/L for YE. The solution to the maximal lactic acid production was 36.74 g/L for CDS and 7.58 g/L for YE. Last, the solution to the simultaneous maximal production of nisin and lactic acid was 37.08 g/L for CDS and 7.34 g/L for YE. The results also reveal that the predicated values of nisin and lactic acid under these three conditions have no significant difference.

In order to confirm the optimal conditions obtained from the statistically based experimental designs, a verification experiment under the conditions of 37.0 g/L CDS and 7.5 g/L YE was conducted. After 24 h of fermentation 85.0 mg/L of nisin and 19.3 g/L of lactic acid were obtained. The result was very close to the predicted value. In addition, the nisin result also agreed well with the "ceiling concentration" of nisin previously reported by other researchers *(16,17)*. Therefore, the optimal conditions predicted from the statistically based experimental designs were valid. In brief, we aimed at verifying the feasibility of using CDS as a nutrient supplement for nisin and lactic acid coproduction by *L. lactis* from cheese whey. The results indicated that CDS is a good source of nutrients essential for nisin and lactic acid cofermentation; however, only a limited amount of CDS can be added for this purpose. The optimal concentration of CDS in fermentation medium was 37.0 g/L, when 7.5 g/L of YE was applied as auxiliary nutrient.

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