Strain Selection and Optimization of Mixed Culture Conditions for *Lactobacillus pentosus* K1-23 with Antibacterial Activity and *Aureobasidium pullulans* NRRL 58012 Producing Immune-Enhancing β -Glucan^S

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Received: January 26, 2018

Revised: March 2, 2018

Accepted: March 8, 2018

First published online
March 16, 2018

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^SSupplementary data for this
paper are available on-line only at
<http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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Lactobacillus pentosus K1-23 was selected from among 25 lactic acid bacterial strains owing to its high inhibitory activity against several pathogenic bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *S. gallinarum*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, and *Listeria monocytogenes*. Additionally, among 13 strains of *Aureobasidium* spp., *A. pullulans* NRRL 58012 was shown to produce the highest amount of β -glucan ($15.45 \pm 0.07\%$) and was selected. Next, the optimal conditions for a solid-phase mixed culture with these two different microorganisms (one bacterium and one yeast) were determined. The optimal inoculum sizes for *L. pentosus* and *A. pullulans* were 1% and 5%, respectively. The appropriate inoculation time for *L. pentosus* K1-23 was 3 days after the inoculation of *A. pullulans* to initiate fermentation. The addition of 0.5% corn steep powder and 0.1% FeSO_4 to the basal medium resulted in the increased production of lactic acid bacterial cells and β -glucan. The following optimal conditions for solid-phase mixed culture were also statistically determined by using the response surface method: 37.84°C , pH 5.25, moisture content of 60.82%, and culture time of 6.08 days for *L. pentosus*; and 24.11°C , pH 5.65, moisture content of 60.08%, and culture time of 5.71 days for *A. pullulans*. Using the predicted optimal conditions, the experimental production values of *L. pentosus* cells and β -glucan were $3.15 \pm 0.10 \times 10^8$ CFU/g and $13.41 \pm 0.04\%$, respectively. This mixed culture may function as a highly efficient antibiotic substitute based on the combined action of its anti-pathogenic bacterial and immune-enhancing activities.

Keywords: *Aureobasidium*, immune-enhancing β -glucan, *Lactobacillus*, anti-pathogenic activity, mixed culture

Introduction

Antibiotics are chemotherapeutic agents used for the clinical management of infectious diseases in humans and animals. Antibiotics have been added to animal feed to enhance agricultural and aquatic productivity. However, owing to increased consumer concerns regarding food safety and the appearance of super-bacteria, the use of antibiotics in animal feed has been regulated. Recently, the use of antibiotics to promote animal growth was completely

banned in Korea. However, the regulation of antibiotic use in animal feed has led to a decrease in productivity due to increased mortality; therefore, the development of antibiotic alternatives is essential.

Probiotics promote beneficial microbial growth while inhibiting pathogenic microorganisms, promote animal growth, and improve feed efficiency. Probiotics also prevent intestinal diseases in young animals owing to intestinal microbial competition, reduce pathogenic microorganisms, and improve immune function [1, 2]. Lactic acid bacteria

(LAB) are the most widely used probiotics, and *Lactobacillus* and *Bifidobacterium* are the safest species and have been studied by many researchers [3]. The antimicrobial effects of LAB include the production of low pH, organic acids, bacteriocins, carbon dioxide, ethanol, diacetyl, low-molecular-weight antimicrobials, low reduction potential, nutrient depletion, and dominance [4].

Physiologically active polysaccharides produced by bacteria, yeast, and molds are used in a variety of industrial applications, including pharmaceuticals, cosmetics, and the food industry, and many polysaccharides have been reported to exhibit antioxidant, antitumor, antiviral, anti-inflammatory, and immune system-enhancing effects [5–7]. β -Glucan, a well-known physiologically active polysaccharide, is a chain-type polysaccharide in which glucose molecules are linked by β -D-glycosidic bonds. β -Glucan is mainly present in the form of β -(1,3)-glucan or β -(1,6)-glucan in yeast and mushrooms and in the form of β -(1,3)-glucan and β -(1,4)-glucan in oat and barley [8]. β -(1,3),(1,6)-Glucan is a branched β -glucan form in which a glucose(s) is connected to the main chain by a β -(1,6)-glycosidic bond. β -(1,3),(1,6)-Glucan exerts antitumor [9], anti-infectious disease [10], and anti-inflammation [11] effects by regulating the immune system.

Aureobasidium pullulans, also known as black yeast, is a polymorphic fungus that is widespread in nature. *A. pullulans* produces complex exopolysaccharides, including pullulan (α -glucan), which is used in the pharmaceutical industry [12]. β -(1,3),(1,6)-Glucan derived from *A. pullulans* cultures has been recognized as a food additive and is consumed as a health-promoting food in many countries [13]. When producing health supplements containing β -(1,3),(1,6)-glucan from other organisms, an extraction process, such as hot water extraction, is required. However, β -(1,3),(1,6)-glucan produced by *A. pullulans* is water-soluble and therefore can be used as a food additive without requiring an extraction process. β -(1,3) or (1,6)-glucan produced from mushrooms has a high molecular mass (~2,000 kDa) and is insoluble in water [14], but β -(1,3),(1,6)-glucan of *A. pullulans* consists of small molecules approximately 100 kDa in size that are soluble in water [15].

In addition to the advantages described above, β -(1,3),(1,6)-glucan produced by *A. pullulans* has nearly the same efficacy as the same type of glucan produced in other organisms. For example, β -(1,3),(1,6)-glucan produced by *A. pullulans* has been shown to be effective against cancer [16, 17] and to demonstrate anti-allergy [6] and influenza-preventing activities [13].

Existing technology has been effective for commercializing

a variety of antibiotic substitute materials such as enzymes, probiotics, acidifiers, and plant extracts. However, single materials have limited efficacy and have not shown good results. Therefore, in the present study, we combined two different materials with different modes of action against pathogens to develop a more efficient antibiotic substitute. In the present study, we selected a LAB strain with high anti-pathogenic activity and an *A. pullulans* strain producing a high amount of immune-enhancing β -glucan. The optimal culture conditions for solid-phase mixed culture of these two different microorganisms (a bacterium and a yeast) were examined. This mixed culture may function as a highly efficient antibiotic substitute, based on the combined effects of its anti-pathogenic and immune-enhancing activities.

Materials and Methods

LAB and *A. pullulans* Strains

The 25 LAB strains exhibiting high antibacterial activity against various pathogenic bacteria were our laboratory stock cultures. These 25 LAB were previously selected for their anti-pathogenic bacterial activity from among 800 isolates derived from natural sources. The 13 strains of *A. pullulans* used in this study are shown in Table 1.

Pathogenic Bacteria and Cultures

Different pathogenic bacteria and their respective culture media (shown in parenthesis) used in the study included *Escherichia coli* (Luria-Bertani broth), *Salmonella typhimurium*, *S. gallinarum* (tryptic soy broth), *Staphylococcus aureus* (nutrient broth), *Pseudomonas aeruginosa* (nutrient broth), *Clostridium perfringens* (reinforced clostridial medium), and *Listeria monocytogenes* (brain heart infusion medium); all media were BD Difco (USA) products. The bacteria were cultured in 100 ml of liquid medium in a 250-ml flask at 37°C for 1 day.

Minimum Inhibitory Concentration (MIC)

LAB were inoculated into MRS broth and cultured under anaerobic incubation at 37°C for 1 day. The cultures were centrifuged at 9,000 $\times g$, the supernatants were filtered through a 0.45- μm syringe filter, and the filtrates were twice subjected to serial dilution with MRS broth. Each pathogenic bacterium was cultured in broth at 37°C for 1 day, diluted with physiological saline, and adjusted to 1×10^6 CFU/ml. Two hundred microliters of a mixed solution consisting of 10% diluted LAB culture filtrate, 5% pathogenic bacterial culture (1×10^6 CFU/ml), and 85% culture medium for each pathogen was placed in a 96-well plate and incubated at 37°C for 1 day under aerobic condition. The MIC (concentration of LAB culture filtrate in 200 μl of total mixed solution) represented the minimum concentration of the diluted solution of the LAB culture filtrate in which pathogenic bacteria did not proliferate.

Table 1. List of *Aureobasidium* strains used in this study.

Strain	Scientific name	Source
YH	<i>A. pullulans</i>	Laboratory stock culture
CU26	<i>A. pullulans</i>	H. Punnapayak & S. Prasongsuk Chulalongkorn Univ. Thailand
CU30	<i>A. pullulans</i>	H. Punnapayak & S. Prasongsuk Chulalongkorn Univ. Thailand
YR	<i>A. pullulans</i>	Laboratory stock culture
KACC 41291	<i>A. pullulans</i>	Korean Agricultural Culture Collection
KACC 43382	<i>A. pullulans</i>	Korean Agricultural Culture Collection
KCCM 12017	<i>A. pullulans</i>	Korean Culture Center of Microorganisms
NRRL 58012	<i>A. pullulans</i>	ARS Culture Collection (NRRL), USA
NRRL 58013	<i>A. pullulans</i>	ARS Culture Collection (NRRL), USA
NRRL 58522	<i>A. pullulans</i>	ARS Culture Collection (NRRL), USA
NRRL 58539	<i>Aureobasidium</i> sp.	ARS Culture Collection (NRRL), USA
NRRL 58543	<i>Aureobasidium</i> sp.	ARS Culture Collection (NRRL), USA
NRRL 62100	<i>A. pullulans</i>	ARS Culture Collection (NRRL), USA

***A. pullulans* Culture**

The liquid medium for *A. pullulans* was glucose-sucrose (GS) medium containing 2.5% glucose, 2.5% sucrose, 0.2% ascorbic acid, 0.1% K_2HPO_4 , 0.2% $MgSO_4 \cdot 7H_2O$, and 1.0% corn steep liquor. One loopful of culture was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of GS and cultured for 5 days at 25°C and 200 rpm. The solid medium for β -glucan production consisted of 8% molasses, 0.2% ascorbic acid, 3% amino acid fermentation waste (Daesang co., Ltd, Korea), and 88.8% soybean powder. GS broth cultured for 3 days was inoculated into solid medium, mixed well, and cultured at 25°C for 5 days.

Analysis of Glucan in Solid Culture Medium

Total glucan and β -glucan contents were measured in solid culture using a β -glucan assay kit (Megazyme International Ireland Ltd., Ireland). For pretreatment, the solid culture was mixed well, and 100 mg of culture was added to a conical flask with 1.5 ml of 37% hydrochloric acid. The mixture was stirred for 45 min in a 37°C water bath, and then 10 ml distilled water was added and hydrolysis was carried out for 2 h at 100°C. The hydrolyzate was cooled down and neutralized by adding 10 ml of 2 N KOH and centrifuged at 3,000 $\times g$ to obtain a supernatant. For total glucan analysis, 0.1 ml of exo-1,3- β -glucosidase plus β -glucosidase was added to 0.1 ml of pretreated supernatant, and the reaction was carried out at 40°C for 60 min to decompose total glucan into glucose. Glucose was assayed by the DNS method [18], and the total amount of glucan was calculated from the amount of glucose produced.

For α -glucan analysis, 100 mg of well-mixed solid culture was added to a test tube, followed by the addition of 2 ml of 2 M KOH, and the mixture was stirred for 20 min; then, 8 ml of 1.2 M sodium acetate buffer (pH 3.8) and 0.2 ml of amyloglucosidase plus

invertase were added. The reaction was carried out in a 40°C water bath for 30 min. The reaction solution was centrifuged, the amount of glucose in the supernatant was quantified by DNS, and the amount of α -glucan was calculated from the produced glucose. The β -glucan content was calculated by subtracting the α -glucan content from the total glucan content.

Identification of Microorganisms

The final selected LAB strain was identified using 16S rRNA gene sequencing. PCR (PCR system 2700; GeneAmp Co., Singapore) was performed using 27F (5'-GATTGGATCCTGGCTCAG-3') and 1492R (5'-AAGGAGGGGATCCAGCC-3') as PCR primers. The PCR mixture consisted of 2 μ l of template DNA, 1 μ l of 27F primer, and 1 μ l of 1492R primer in a total volume of 20 μ l. The following PCR conditions were used: initial denaturation at 94°C for 2 min, denaturation at 94°C for 20 sec, annealing at 60°C for 20 sec, and extension at 72°C for 1 min and 30 sec. The PCR was performed for 30 cycles, after which final extension was carried out at 72°C for 5 min. The PCR products were purified using a QIA Quick PCR purification kit (Qiagen, USA), and DNA sequencing analysis was performed by a commercial company (Biomedic Co., Ltd, Korea). An NCBI BLAST homology search (BLASTN) [19] using the obtained sequences was used to identify the LAB.

For yeast strain identification, genomic DNA was extracted [20], and ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers were used for PCR amplification of the ITS region [21]. The DNA was amplified using the procedure of Gardes and Bruns [22]. The purification, DNA sequencing analysis of the PCR products, and subsequent identification were performed in the same manner as described for LAB.

Solid-Phase Mixed Culture of LAB and *A. pullulans*

LAB was cultured in MRS broth at 37°C for 1 day, and *A. pullulans* was grown in GS broth at 25°C for 5 days. A 1% LAB culture and a 5% *A. pullulans* culture were inoculated into solid medium and incubated at 25°C for 5 days. The LAB numbers and β -glucan content were measured.

Inoculation Time and Optimization of β -Glucan Production

Considering the short culture period of LAB, we first inoculated *A. pullulans* into solid medium and later inoculated the LAB at 1-day intervals to find the optimal LAB inoculation point for the LAB cells and β -glucan production.

Effects of Medium Components on LAB Cells and β -Glucan

In our previous study, the concentrations of amino waste solution and soybean powder in basic solid medium (8% molasses, 0.2% ascorbic acid, 3% amino acid waste solution, 88.8% soybean powder, and 70% moisture content) were optimized [23]. In this

study, different nitrogen sources, minerals, and concentrations of ascorbic acid were added to the basic solid medium for inoculation and cultured at 25°C for 5 days; then, LAB cell counts and β -glucan contents were determined.

Nitrogen sources: 0.5% NaNO₃, NH₄Cl, urea, soybean meal, corn steep powder (CSP), or skim milk were added to the basic solid medium.

Minerals: After 0.5% CSP was added to the basic solid medium, 0.1% MgSO₄, MnCl₂, CuSO₄, FeSO₄, ZnSO₄, or CaCl₂ was added to the medium.

Ascorbic acid: 0.0%, 0.1%, 0.2%, 0.3%, or 0.4% ascorbic acid was added to the basic medium containing 0.5% CSP and 0.1% FeSO₄.

Optimization of Mixed Culture Conditions by Using a Statistical Method

To optimize the culture conditions for the solid medium, statistical optimization was performed by varying the moisture content, temperature, pH, and culture days. An experiment was

Table 2. Minimum inhibitory concentrations (MIC, %) of various LAB culture supernatants.

Strain	MIC (%)						
	<i>E. coli</i>	<i>S. typhimurium</i>	<i>S. gallinarum</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. perfringens</i>	<i>L. monocytogenes</i>
K1-1	5	5	1.25	10	1.25	10	10
K1-3	2.5	10	1.25	10	1.25	10	10
K1-5	1.25	10	5	10	5	10	10
K1-6	5	5	2.5	10	1.25	10	5
K1-7	5	2.5	1.25	5	1.25	5	10
K1-8	5	10	5	10	5	10	10
K1-9	5	5	2.5	10	5	5	5
K1-10	2.5	10	1.25	10	5	10	>10
K1-11	1.25	5	2.5	5	5	2.5	5
K1-13	5	2.5	5	10	5	10	10
K1-14	5	10	5	10	40	10	10
K1-15	5	2.5	5	10	2.5	10	>10
K1-16	5	5	5	5	2.5	10	5
K1-17	2.5	10	1.25	10	1.25	10	10
K1-18	5	10	1.25	5	1.25	5	10
K1-19	2.5	2.5	1.25	5	1.25	2.5	5
K1-20	2.5	10	5	10	2.5	5	5
K1-21	5	10	2.5	10	1.25	10	10
K1-22	5	10	2.5	5	1.25	5	5
K1-23	1.25	2.5	1.25	5	1.25	2.5	10
K2-4	10	10	5	5	1.25	2.5	10
K2-5	10	10	5	5	5	10	10
K4-4	10	10	10	10	10	10	10
K5-3	10	10	10	10	10	5	10
K5-5	10	10	2.5	5	5	10	10

performed using the response surface methodology (RSM) with four factors (Table S1). A central composite design (CCD) was used to determine the design points. Sixteen points for factorial experiments, eight points for the axis point, and six points for the central point were required by the CCD design.

The second-order regression model is as follows:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{i < j} \beta_{ij} X_i X_j \quad (1)$$

where Y is the estimated response value, X_i is the coded variable, β_0 is the intercept, β_i is the first-order coefficient, β_{ii} is the second-order coefficient, and β_{ij} is the interaction coefficient between X_i and X_j .

On the other hand, note that X_i has the following relationship:

$$X_i = \frac{x_i - x_0}{\Delta x_i}$$

where X_i is the coded value, x_i is the actual value, x_0 is the center value, and Δx_i is the increment value. Eq. (1) shows a regression model with coded variables. X_i is encoded into one of the following five values: ± 2 , ± 1 , and 0. Table 2 shows the actual and coded values for the four factors used in this study. The optimal experimental levels for LAB cell numbers and β -glucan production were investigated by using RSM.

Results and Discussion

Selection of LAB for Anti-Pathogenic Activity

The MICs of 25 LAB strains were examined, and the results are shown in Table 2. Fifteen strains (K1-1, K1-3, K1-5, K1-6, K1-7, K1-10, K1-11, K1-13, K1-15, K1-17, K1-18, K1-19, K1-21, K1-22, and K1-23) were selected on the basis of their MICs, and these strains were used in mixed culture with *A. pullulans* for further selection.

Selection of *A. pullulans* Producing β -Glucan

Thirteen strains of *A. pullulans* were examined for their β -glucan production on solid culture medium, and *A. pullulans* NRRL 58012 produced the highest amounts of β -glucan ($15.45 \pm 0.07\%$) and was selected (Table S2).

Final Selection of LAB for Mixed Culture

In a solid-phase mixed culture with *A. pullulans*, the highest amounts of β -glucan (12.4–12.7%) were produced by K1-13, K1-19, and K1-23 (Table 3). In mixed culture, LAB K1-15 produced the highest cell numbers (2.75×10^8 CFU/g), but its β -glucan production was low (9.6%). In all mixed cultures, β -glucan production was less than 14.4% of the control with no LAB inoculation. LAB K1-23 was selected because this strain satisfied the requirements for high production of

Table 3. Production of LAB cells and β -glucan by solid-phase mixed cultures of various LAB strains^a and *A. pullulans* NRRL 58012^b.

LAB strain	LAB viable cell no. ($\times 10^8$ CFU/g)	Concentration (% w/w)		
		Total glucan	α -Glucan	β -Glucan
Control	0	21.1	6.7	14.4
K1-1	2.21	15.6	9.5	6.1
K1-3	1.32	20.3	8.6	11.6
K1-5	2.04	17.8	9.3	8.5
K1-6	2.57	13.2	11.3	1.9
K1-7	0.35	16.5	10.3	6.2
K1-10	1.21	19.2	8.7	10.5
K1-11	1.38	20.0	9.2	10.8
K1-13	0.93	21.2	8.5	12.7
K1-15	2.75	19.5	9.9	9.6
K1-17	2.42	19.4	9.5	9.9
K1-18	1.66	18.9	10.2	8.7
K1-19	2.01	20.5	8.1	12.4
K1-21	1.36	19.7	9.5	10.2
K1-22	1.75	20.1	8.8	11.3
K1-23	2.35	20.8	8.3	12.5

^aLAB strains were grown in MRS broth for 24 h at 37°C.

^b*A. pullulans* NRRL 58012 was grown in glucose-sucrose broth for 24 h at 25°C.

both LAB cell numbers (2.35×10^8 CFU/g) and β -glucan (12.5%). LAB K1-23 was identified as *Lactobacillus pentosus*. *L. pentosus* was first isolated from human feces and has been used to produce probiotics [24] as well as bacteriocins and inhibits pathogens such as *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [25].

Effects of Inoculum Size on LAB Cell Numbers and β -Glucan Production in Mixed Culture

The effects of inoculum size on *L. pentosus* K1-23 cell numbers and β -glucan production by *A. pullulans* NRRL 58012 in mixed solid-phase culture were examined, and the results are shown in Table S3. With different inoculum sizes (1%, 5%, 10%) of *L. pentosus*, the average number of LAB cells was $2.67 \pm 0.19 \times 10^8$ CFU/g, and differences in LAB cell production were not significant. Whereas 10% *L. pentosus* inoculum produced a lowered amount of β -glucan, there was no difference in β -glucan production between 1% and 5% *L. pentosus* inoculums. With different inoculum sizes (1%, 5%, 10%) of *A. pullulans*, differences in LAB cell production were not observed. Our previous statistical study showed that inoculum size did not significantly

affect β -glucan production by *A. pullulans* [23]. The best combination of inocula was 1% *L. pentosus* and 5% *A. pullulans*, from which 2.51×10^8 CFU/g and 12.9% β -glucan were produced, respectively.

Differential Inoculation Times for *L. pentosus* K1-23 in a Mixed Culture

Thus far, in these mixed culture experiments, the two microorganisms were inoculated at the beginning of the mixed culture and incubated for 5 days. Since *L. pentosus* K1-23 cells grow significantly faster and its culture time is shorter than that of *A. pullulans* NRRL 58012, *L. pentosus* could be inoculated later than *A. pullulans*. The optimal inoculation time for *L. pentosus* was determined by inoculating *L. pentosus* at 1-day intervals, and the results are shown in Fig. 1.

When the two strains were inoculated simultaneously at day 0, the final number of *L. pentosus* cells was 2.52×10^8 CFU/g, followed by 2.38×10^8 CFU/g on day 1, 2.31×10^8 CFU/g on day 2, 2.35×10^8 CFU/g on day 3, and 2.21×10^8 CFU/g on day 4; there was no significant difference in the number of final *L. pentosus* cells with different inoculation times. With earlier inoculation (day 0, 1, and 2) of *L. pentosus*, β -glucan production was significantly reduced prior to 120 h of culture. However, after 120 h of culture, 12.72–13.05% β -glucan was obtained, and the difference was not significant for different inoculation times. With *L. pentosus* inoculation on the 3rd or 4th day of *A. pullulans*

Fig. 1. Effect of *L. pentosus* K1-23 inoculation time on β -glucan production by *A. pullulans* NRRL 58012 in the solid-phase mixed culture.

culture, maximum β -glucan production was achieved at 108 h instead of 120 h with earlier inoculation times; thus, it was possible to shorten the culture time by 12 h.

Optimization of Medium Composition for Mixed Culture

Nitrogen source. With the addition of different nitrogen sources, the average total number of cells for *L. pentosus* K1-23 was $2.52 \pm 0.06 \times 10^8$ CFU/g, and there were almost no differences in LAB cell numbers among the different nitrogen sources. β -Glucan production was $13.0 \pm 0.15\%$ in the control group without the addition of nitrogen sources, and $13.2 \pm 0.17\%$ and $13.1 \pm 0.10\%$ when CSP and skim milk were added, respectively (Fig. 2A). CSP was selected because it produced relatively higher β -glucan levels and is a cheap medium component.

Minerals. β -Glucan production was slightly higher with the addition of CuSO_4 ($13.3 \pm 0.07\%$), FeSO_4 ($13.4 \pm 0.14\%$), and ZnSO_4 ($13.3 \pm 0.14\%$) than in control culture ($13.2 \pm 0.17\%$) (Fig. 2B). The number of *L. pentosus* cells after the addition of different minerals varied from 2.33 – 2.93×10^8 CFU/g. MgSO_4 , MnCl_2 , and CaCl_2 produced relatively higher LAB cell numbers (2.80×10^8 , 2.93×10^8 , and 2.91×10^8 CFU/g, respectively), but β -glucan production was

Fig. 2. Effects of different nitrogen sources (A) and minerals (B) on β -glucan production by *A. pullulans* NRRL 58012 in the solid-phase mixed culture with *L. pentosus* K1-23.

SML, soy bean meal; CSP, corn steep liquor; SM, skim milk; Mg, MgSO_4 ; Mn, MnCl_2 ; Cu, CuSO_4 ; Fe, FeSO_4 ; Zn, ZnSO_4 ; Ca, CaCl_2 .

lower than that of the control group. We selected FeSO_4 , which produced the highest LAB cell numbers (2.68×10^8 CFU/g) as well as β -glucan ($13.4 \pm 0.14\%$). Gao *et al.* [26] reported that K_2HPO_4 is the most important mineral for cell growth and pullulan production.

Ascorbic acid. Different concentrations of ascorbic acid showed no difference in β -glucan production ($13.39 \pm 0.01\%$) and LAB cell numbers ($2.63 \pm 0.05 \times 10^8$ CFU/g).

Optimization of Culture Conditions Using a Statistical Method

To determine the optimal conditions for solid-phase mixed culture, second-order regression models were generated by varying the temperature (X_1), pH (X_2), moisture content (X_3), and culture time (X_4). The experimental levels of LAB cell numbers and β -glucan production from the CCD experiment are shown in Table S4.

The second-order regression model for total LAB cell

number was derived from Table S4 and is as follows:

$$Y = 2.563 + 0.188X_1 - 0.010X_2 + 0.043X_3 + 0.485X_4 - 0.025X_1X_2 - 0.000X_1X_3 + 0.010X_1X_4 + 0.018X_2X_3 - 0.015X_2X_4 + 0.005X_3X_4 - 0.021X_1^2 - 0.103X_2^2 - 0.108X_3^2 - 0.253X_4^2$$

Fig. 3 is a response surface plot of the second-order regression. It is composed of six plots showing that *L. pentosus* cell production is affected by temperature and pH, temperature and moisture content, temperature and culture time, pH and moisture content, and moisture content and culture time; there was a quadratic curve effect for the varying levels of each factor, leading to the optimal point. The optimal point derived by the surface response model equation was 37.84°C, pH 5.25, moisture content of 60.82%, and 6.08 days, and the expected maximum value for *L. pentosus* cell numbers was 3.32×10^8 CFU/g.

The second-order regression model for β -glucan

Fig. 3. Response surface plots and corresponding contour plot for the cell number of *L. pentosus* K1-23 in a mixed culture.

production is as follows:

$$Y = 13.172 - 0.026X_1 - 0.080X_2 + 0.066X_3 + 0.580X_4 + 0.015X_1X_2 + 0.013X_1X_3 + 0.004X_1X_4 + 0.010X_2X_3 - 0.026X_2X_4 + 0.016X_3X_4 - 0.046X_1^2 - 0.144X_2^2 - 0.258X_3^2 - 0.414X_4^2$$

As shown in Fig. 4, there was a quadratic curve effect for the varying levels of each factor, leading to the optimal point. The optimal point derived by the surface response model equation to maximize β -glucan production was 24.11°C, pH 5.65, moisture content of 60.08%, and a culture time of 5.71 days, at which the expected maximum β -glucan production was 13.40%. Gao *et al.* [26] reported that the optimal pH for cell growth and pullulan production by *A. pullulans* was 6.0, which is close to the optimal pH of 5.65 identified in our study for β -glucan production.

To compare the theoretical predicted values with actual

production amounts, LAB cells and β -glucan production were tested for each optimal condition (Table 4). Using the optimal conditions for *L. pentosus* cell production (37.84°C, pH 5.25, moisture content of 60.82%, and 6.08 days), $3.15 \pm 0.10 \times 10^8$ CFU/g was achieved, which was close to the expected production value of 3.32×10^8 CFU/g. Using the optimal conditions for β -glucan (24.11°C, pH 5.65, moisture content of 60.08%, and culture time of 5.71 days), actual β -glucan production was $13.41 \pm 0.04\%$, which was nearly the same as the expected value of 13.40%.

In this study, we selected *L. pentosus* K1-23, which greatly inhibits various pathogenic bacteria, as well as *A. pullulans* NRRL 58012, which produces high amounts of immune-enhancing β -glucan, and optimized the medium composition and culture conditions for solid-phase mixed culture to achieve maximal production of LAB cell numbers and β -glucan. This mixed culture could be used as a highly

Fig. 4. Response surface plots and corresponding contour plot for β -glucan production by *A. pullulans* NRRL 58012 in a mixed culture.

Table 4. Predicted and experimental values of LAB cell numbers and β -glucan produced by a mixture culture of *L. pentosus* K1-23 and *A. pullulans* NRRL 58012.

Run no.	Temperature (°C)	pH	Moisture (%)	Culture time (day)	Predicted values		Experimental values	
					LAB cell number ($\times 10^8$ CFU/g)	β -Glucan (%)	LAB cell number ($\times 10^8$ CFU/g)	β -Glucan (%)
1	37.84	5.25	60.82	6.08	3.32	-	3.15 $\pm$ 0.10	11.86 $\pm$ 0.21
2	24.11	5.65	60.08	5.71	-	13.40	2.68 $\pm$ 0.06	13.41 $\pm$ 0.04

efficient antibiotic substitute based on the combined effects of its anti-pathogenic and immune-enhancing activities.

Acknowledgments

This work (Grant No. C0213060) was supported by the Business for Cooperative R&D between Industry, Academy, and Research Institute funded by the Korea Small and Medium Business Administration in 2013. H.C. Jeong was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2015R1D1A1A09059859). We thank to Dr. H. Punnapayak and S. Prasongsuk at Chulalongkorn University, Thailand for providing us 2 strains of *A. pullulans*.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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