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Production of Biological Control Agent *Bacillus subtilis* B579 by Solid-State Fermentation using Agricultural Residues

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Three different agricultural residues were evaluated as basic substrates for the production of biological control agent (BCA) *Bacillus subtilis* strain B579, by solid-state fermentation (SSF). Among them, wheat bran (WB) was shown to be the most suitable substrate, and an initial moisture content of 57% was determined to be optimal for SSF of strain B579. Moreover, employing the Plackett-Burman and Box-Benhnken experimental design models, nine different supplements were investigated to maximize the cell yield. Results demonstrated that a relatively maximal cell yield of $2.12\pm0.03\times10^{11}$ CFU/g was achievable utilizing WB as the basic medium supplemented with beef extract (21.3 g kg^{-1}), cornmeal (42.6 g kg^{-1}), and soluble starch (23.8 g kg^{-1}).

Key words: Agricultural residues, *Bacillus subtilis* B579, Biological Control agent, Design-Expert, Solid-State Fermentation.

Over the last few decades, the use of biological control agents (BCAs) to protect crops from the damage caused by phytopathogens has become increasingly common owing to their prolonged effects and reduced risk to the ecosystem and human health[1]. Several bacterial genera such as Pseudomonad, Bacillus, Actinomyce and Agrobacteria, have been exploited as BCAs. Among them, spore-forming Bacillus was considered to be a promising BCA candidate, and was extensively studied since the Bacillus spores were convenient for production, application, and preservation[2, 3].

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Conventionally, submerged fermentation (SmF) is the predominantly employed method of BCAs production. However, compared with SmF of BCAs, solid-state fermentation (SSF) of BCAs offers certain advantages including cost efficiency, water and energy saving, reduced waste discharge, and a requirement for simple fermentation devices[3]. Therefore, attention has been focused on the SSF strategy for the production of enzymes, secondary metabolites, as well as BCAs in recent years[4]. The aim of our current work was to investigate the potential application of SSF using agricultural residues as substrates to support the production of *Bacillus subtilis* strain B579. Three types of agricultural residues (wheat bran, soybean residues and mushroom residues), which are inexpensive and easily available in Northern China, were investigated for B579 production, with supplementation of certain materials to improve the cell yield.

MATERIALS AND METHODS

Microorganisms and inocula preparation

The biocontrol strain *B. subtilis* B579 was isolated from vegetable rhizospheres cultivated under greenhouse conditions from Tianjin City[5]. While the strain B579-GFP exhibits the same cultivation pattern as that of the wild-type strain B579, it is easily differentiated from contaminating microorganisms due to its GFP fluorescence and tetracycline resistance (data not shown). Hence, B579-GFP was used in all fermentation protocols in this study. Our previous study demonstrated that the optimal temperature and pH conditions for the cultivation of strain B579-GFP were 37°C and 7.2, respectively (data not shown). Hence, these parameters were adopted for all the SSF protocols in this study.

To prepare inocula, B579-GFP was incubated in liquid Luria-Bertani (LB) media until the OD_{600} reached approximately 1.0, equating to a cell density of approximately 1.7×10^9 CFU ml^{-1}). The culture was diluted to a final cell density of 1.0×10^8 CFU ml^{-1} for use as the inoculum in SSF protocols. In this study, 0.2 ml inocula were transferred into 20 g SSF medium to give an initial cell density of approximately 1.0×10^5 CFU g^{-1}.

Fermentation batches

In this study, the cell yield under SSF conditions was evaluated using three types of substrates, (wheat bran, WB; soybean residues, SR; mushroom residues, MR), and nine supplementary materials (yeast extract, beef extract, peptone, soluble starch, cornmeal, glucose, sucrose, maltose, KH_2PO_4) were added to improve the yield. In SSF protocols, moisture content of the substrates was maintained 57% unless otherwise mentioned.

Optimization of growth parameters

To determine the optimal substrate for B579-GFP cell production, substrates (20 g) were added individually into 250 ml Erlenmeyer flasks, autoclaved, inoculated with B579-GFP, and then incubated for 4 d.

Using WB as the basic substrate, different ratios of SR, MR, or SR plus MR were tested to determine the cell yield during the course of SSF. Effects of the moisture content and the initial cell density on cell yield were also evaluated.

To further improve the cell yield of B579-GFP under SSF conditions, nine different supplements (yeast extract, beef extract, peptone, soluble starch, cornmeal, glucose, sucrose, maltose, KH_2PO_4) were separately added to the incubation medium. The Plackett-Burman screening design was applied to evaluate the effects of these supplements using the software Design-Expert (V8.0.6). The coded levels of variables are shown in Table 1, and the Plackett-Burman experimental designs are listed in Table 2. Based on the results of Plackett-Burman screening (Table 3), experiments of steepest ascent were designed and performed (Table 4). The Box-Benhnken designs for the assigned concentration of three designated factors are shown in Table 5.

Cell quantification of the SSF production

Samples (1 g) were introduced into 50 ml flasks containing 10 ml sterilized water and glass beads, and subjected to shaking at 220 rpm for 20 min to suspend the cells. Then samples were then diluted and plated onto solid LB medium (supplemented with tetracycline, 20 μg ml^{-1}) for cell quantification. The amount of cells was defined as CFU of per gram dry substrate (CFU g^{-1}).

RESULTS AND DISCUSSION

The substrate screening for SSF from agricultural residues

As described in the Materials and methods, the GFP-labeled *B. subtilis* strain B579-GFP was used as a substitute for the wild-type strain B579 in SSF protocols in this study. To determine the influence of different substrates on

Table 1. Coded levels of factors for the Plackett-Burman experimental design

Factors (g·Kg-1)	Symbol	Coded levels	
		-1	1
Beef extract	X1	15	25
Yeast extract	X2	15	25
Peptone	X3	10	17
Maltose	X4	8	12
Cornmeal	X5	30	50
Glucose	X6	6	10
Sucrose	X7	8	16
Soluble starch	X8	15	30
KH_2PO_3	X9	3	5
Dumy1	X10	-1	1
Dumy2	X11	-1	1

Table 2. Plackett-Burman experimental design matrix of variables

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Response $\times 10^{11}$ CFU g^{-1}
1	25.00	25.00	10.00	12.00	30.00	10.00	8.00	15.00	5.00	-1.00	1.00	1.73
2	15.00	15.00	10.00	8.00	30.00	6.00	8.00	15.00	3.00	-1.00	-1.00	1.53
3	15.00	25.00	10.00	12.00	50.00	6.00	16.00	30.00	5.00	-1.00	-1.00	1.87
4	25.00	15.00	17.00	8.00	50.00	6.00	8.00	30.00	5.00	-1.00	1.00	1.79
5	25.00	25.00	17.00	8.00	30.00	6.00	16.00	15.00	5.00	1.00	-1.00	1.62
6	15.00	15.00	10.00	8.00	30.00	10.00	16.00	30.00	5.00	1.00	1.00	1.56
7	25.00	15.00	17.00	12.00	30.00	10.00	16.00	30.00	3.00	-1.00	-1.00	1.73
8	25.00	15.00	10.00	12.00	50.00	6.00	16.00	15.00	3.00	1.00	1.00	1.93
9	15.00	25.00	17.00	12.00	30.00	6.00	8.00	30.00	3.00	1.00	1.00	1.61
10	15.00	15.00	17.00	12.00	50.00	10.00	8.00	15.00	5.00	1.00	-1.00	1.89
11	15.00	25.00	17.00	8.00	50.00	10.00	16.00	15.00	3.00	-1.00	1.00	1.68
12	25.00	25.00	10.00	8.00	50.00	10.00	8.00	30.00	3.00	1.00	-1.00	1.76

Table 3. Analysis of variance (ANOVA) for selected factorial models

Source	Sum of squares	Mean square	F-Value	P-value
Model	0.18	0.060	57.75	< 0.0001
A-Beef extract	0.015	0.015	14.23	0.0055
E-Cornmeal	0.11	0.11	104.81	< 0.0001
H-Soluble starch	0.056	0.056	54.23	< 0.0001

Table 4. Experimental designs and the results of steepest ascent experiments

Step	X_1/g·Kg^{-1}	X_5/g·Kg^{-1}	X_8/g·Kg^{-1}	cell yield/ $\times 10^{11}$cfu g^{-1}
0	24	48	28	1.82
0+1Δ	22	44	25	1.98
0+2Δ	20	40	22	1.89
0+3Δ	18	36	19	1.79
0+4Δ	16	32	16	1.60

Table 5. Assigned concentration of each variable at different levels in the Box-Benhnken experimental design

Factors	Symbol	Code level	
		-1	1
Beef extract/g·Kg^{-1}	X1	21	23
Cornmeal/g·Kg^{-1}	X5	41	47
Soluble starch/g·Kg^{-1}	X8	21	27

cell yields, B579-GFP was cultured under SSF conditions using WB, SR, and MR as basic substrates with various moisture contents (40%, 45%, 50%, 55%, 60%, 65%, and 70%). Figure 1 shows that WB gave rise to the highest cell yield (1.66×10^{11} CFU g^{-1}) compared with SR (1.17×10^{11} CFU g^{-1}) and MR (0.96×10^{11} CFU g^{-1}). The maximum yield of B579-GFP cells occurred on the fourth day using WB as the substrate, one day earlier than that using SR and MR. These results indicated that WB is suitable for use as the basic substrate for B579-GFP SSF cultures, and that the fourth day of culture is the optimal time-point to measure the cell yield in subsequent experiments.

Using WB as the primary substrate, different ratios of SR, MR, or SR plus MR were supplied in different formulations to further improve cell production. The pure WB (formula 1) still yielded the highest cell density (1.69×10^{11} CFU g^{-1}) (Fig. 2). The cell yield of formula 4 (WB: SR: MR = 2: 1: 1) yielded the second highest cell density (1.59×10^{11} CFU g^{-1}), although there was no significant difference between the yields generated using formula 1 and formula 4. Formula 2 (WB: SR

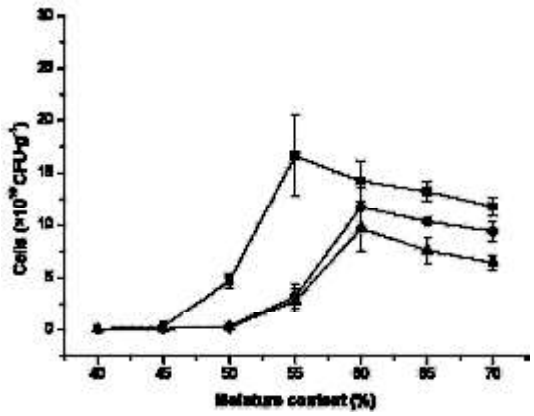

Fig. 1. Effect of moisture content on cell yields using three basic substrates. The influence of moisture content on cell yields using three basic substrate (WB, squares; SR, circles; MR, triangles) was measured. Samples were taken from at least three independent media on the fourth day of culture. Error bars indicate standard deviations.

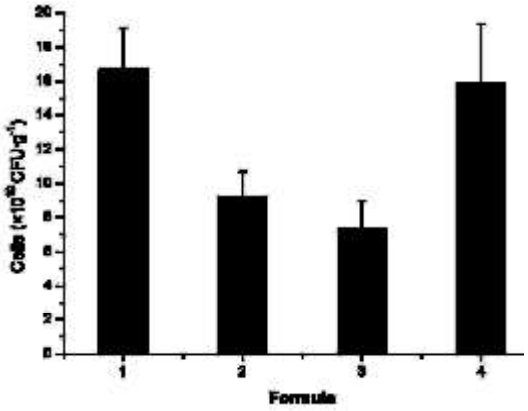

Fig. 2. The cell yields of different substrate formulations.Formula 1, WB; formula 2, WB: SR = 1: 1; formula 3, WB: MR = 1: 1; formula 4, WB: SR: MR = 2: 1: 1. Samples were taken from at least three independent media on the fourth day of culture. Error bars indicate standard deviations.

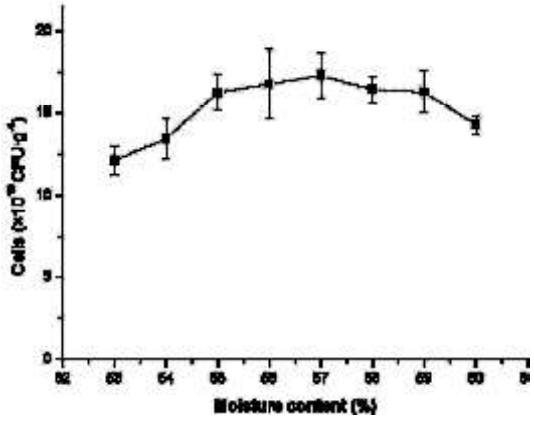

Fig. 3. Effect of moisture content on cell yields The influence of moisture content on cell yields using WB as the basic substrate was measured. Cultures were sampled on the fourth day. Error bars indicate standard deviations.

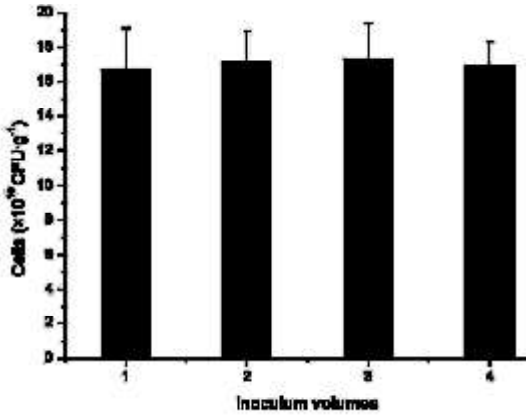

Fig. 4. Effect of inoculum volumes on cell yields Inoculum volumes 1, 2, 3, and 4 represent 100 µl, 200 µl, 500 µl, and 1000 µl, respectively. Experiments were performed in triplicate. Error bars indicate standard deviations.

= 1: 1) and formula 3 (WB: MR = 1: 1) generated lower cell yields (1.52×10^{11} CFU g^{-1} and 1.43×10^{11} CFU g^{-1}, respectively). These results were consistent with previous reports that WB is the optimal substrate for Bacillus SSF in terms of cell or second metabolite yields (3, 6). However, it has also been reported that the maximum yields of lipopeptides or extracellular alkaline protease obtained by Bacillus SSF were generated using lentil husk or SR as basic substrates[4,7]. The optimal fermentation parameters using WB were further analyzed to achieve the maximum cell yield.

Effects of moisture content and initial cell density on cell production

It has been reported that initial moisture content has a significant impact on the cell yield, and only a narrow range of moisture content conditions were shown to promote cell production during Bacillus SSF[8]. Therefore, this parameter was carefully analyzed to maximize the efficiency of cell production. Fig 3 shows the maximum B579-GFP cell production using WB as the basic substrate was achieved with an initial moisture content of 57%. Hence, this value was designated as the fixed

initial moisture content for the subsequent studies.

To investigate the influence of inoculum volumes on the cell yield, different volume of inoculant (100–1000 µl) were added into formula 4, although no significant difference of cell yields were detected (Fig. 4). This result was inconsistent with previous reports of the use of SSF to produce enzymes or secondary metabolites[8].

Effects of supplementary materials on cell production

Our previous study on B579 SmF (data not shown) as well as other studies of SSF demonstrated that supplements such as nitrogen sources, carbon sources or certain chemical compounds remarkably promoted the cell or second metabolite yield[3, 7, 9]. Therefore, the addition of supplementary materials to the basic substrate was investigated for the improvement of B579-GFP yields in this study.

For single factor experiments, coded levels of factors for Plackett-Burman experimental design are shown in Table 1 and the results of this experiment are shown in Table 2. Analysis of variance (ANOVA) was performed to test the significance and adequacy of the model (Table 3). The results demonstrated that the regression model was significant ($P < 0.0001$). The Model F-value of 57.75 implied the model was significant, and a "Model F-Value" occurred due to noise only at a probability of 0.01%. Factors A, E, and H were significant model terms. The steepest ascent experiments were then performed and the results demonstrated an obvious increase in cell yields from step 0 to step 0+1Δ, and a decrease from step 0+1Δ to step 0+2Δ. These results indicated that the optimal amount of supplemental ingredient for the SSF formula was between step 0+1Δ and 0+2Δ, thus 0+1Δ was designated as the central point for subsequent experiments (Table 4). The Box-Benhnken experiments were performed to determine the optimal amount of beef extract, cornmeal, and soluble starch for use in SSF cultures (Table 5). The results of these experiments demonstrated that the predicted maximum cell yield was 2.14×10^{11} CFU g^{-1}, and the optimal amounts of beef extract, cornmeal, and soluble starch were 21.3 g kg^{-1}, 42.6 g kg^{-1}, and 23.8 g kg^{-1}, respectively. The actual cell yield with optimized growth parameters was $(2.12\pm0.03)\times10^{11}$ CFU g^{-1}, which was consistent with the predicted value.

Generally, the results of our study, as well as those reported elsewhere, demonstrated that agricultural residues are suitable for use as the medium for the bacterial SSF process to improve the production efficiency of cells or secondary metabolites. In particular, these materials have the advantage of being inexpensive, abundant, and easily available[3,8,10]. Moreover, the Plackett-Burman and Box-Benhnken experimental design models could be powerful tools for efficient key factor screening in a multivariable system[11,12,13]. Our results demonstrated that a maximum cell yield of *B. subtilis* SSF (strain B579) cultured on a laboratory scale using agricultural residues could reach $2.12\pm0.03\times10^{11}$ CFU g^{-1}. Compared with the cell yeild ($1.86\pm0.01\times10^{10}$ CFU ml^{-1}, unpublished data) of submerged fermentation process, the cell yeild of SSF process was improved about 10 times for *B. subtilis* strain B579 production, which indicated that using agricultural residues to produce BCA of *Bacillus subtilis* strain B579 could be a promising approach on a industrial level. However, since scaling-up of SSF still represents a "bottleneck" for the production[14], future research should be concentrated on the optimization of SSF parameters towards large-scale commercial applications.

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