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Use of surface response methodology to optimize culture conditions for iturin A production by *Bacillus subtilis* in solid-state fermentation

Ing-Lung Shih [a], Chia-Yu Kuo [b], Feng-Chia Hsieh [c], Suey-Sheng Kao [c], Chienyan Hsieh [d,*]

[a] *Department of Environmental Engineering, DaYeh University, Changhwa 51591, Taiwan*
[b] *Department of Bioindustry Technology, DaYeh University, Changhwa 51591, Taiwan*
[c] *Biopesticides Division, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture, Taichung 41358, Taiwan*
[d] *Department of Biotechnology, National Kaohsiung Normal University, Kaohsiung 824, Taiwan*

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Abstract

Response surface methodology (RSM) was employed to optimize the cultivation conditions of *Bacillus subtilis* S3 for the enhancement of iturin A, a lipopeptide antibiotic used as biological pesticide, production in solid-state fermentation (SSF). The statistic experimental model predicted a maximum iturin production of 11.435 mg/g-wet solid material. Verification of the calculated maximum was done with experiments that were performed in the culture media representing the optimum combination found, and the iturin A production of 11.447 mg/g-wet solid material (average of three repeats) was obtained when *B. subtilis* S3 was cultivated at 25 °C for 5 days in solid fermentation containing high gluten flour 10 g and rice bran 50 g in addition to glucose 1.15%, KH_2PO_4 1.27 mM, $MgSO_4$ 5.08 mM, peanut oil 1.01%, inoculum 19.49% and water content 44.97%. The iturin A production by *B. subtilis* S3 was increased significantly by 23%, from 9.26 mg/g-wet solid material to 11.447 mg/g-wet solid material when the strain was cultivated in the optimal medium developed by surface response methodology, as compared to medium conventionally developed by one-factor-at-a-time. The yield of iturin A (11.447 mg/g-wet solid material, with 45% moisture content) produced by *B. subtilis* S3 reported in this study is the highest reported to date for *B. subtilis* species in SSF. In addition, the use of rice bran as a substrate in solid-state fermentation for iturin A production by *B. subtilis* is unique.

Keywords: Iturin A; *Bacillus subtilus*; Surface response methodology; Fermentation; Medium optimization

1. Introduction

Synthetic chemical fungicide has long been served as power agents for reducing the incidence of plant disease; however, they are costly, can cause environmental pollution, and may induce pathogen resistance. Therefore, the conventional use of chemical pesticides has been seriously questioned (Mendgen *et al.*, 1992; Spurrier, 1990). Because of the limitation of chemical fungicide, biological control—the use of microorganism or its secretion to prevent plant disease offers an attractive alternative or supplement to pesticide and genetic resistance for the management of plant disease without the negative aspects of chemical control. The biological control of plant pathogens has become an important aspect of the sustainable agriculture.

In the past few years, numerous microorganisms with antifungal activities and their antifungal factors have been identified (Akihiro *et al.*, 1993; Lim *et al.*, 1991; Lorito *et al.*, 1993; Robert and Selitrennikoff, 1986, 1988; Silo-Suh *et al.*, 1994); in addition, the mechanisms by which microorganisms inhibit growth of potentially pathogenic fungi have been demonstrated (Elad *et al.*, 1982; Howell and Stipanovic, 1980; Lim *et al.*, 1991; Mauch *et al.*, 1988; Phae *et al.*, 1992; Robert and Selitrennikoff, 1988; Silo-Suh *et al.*, 1994). One candidate of microbial control agents is *Bacillus subtilis*, a representative Gram-positive soil bacterium (Asaka and Shoda, 1996; Leclere *et al.*, 2005; Ongena *et al.*, 2005). Till now, several *B. subtilis* were isolated to suppress growth of plant pathogens; such strains and their derivatives were found to have broad suppressive abilities over a variety of plant pathogens in vitro (Phae and Shoda, 1990; Phae *et al.*, 1990) by producing the lipopeptide antibiotics iturin A and surfactin (Asaka and Shoda, 1996; Hiraoka *et al.*, 1992).

* Corresponding author. Tel.: +886 7 7172930x7317; fax: +886 7 6051353.
E-mail address: mch@nknu.edu.tw (C. Hsieh).

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Nomenclature

ANOVA	analysis of variance
CCD	central composite design
D-Asn	D form asparagine
D-Tyr	D form tyrosine
HPLC	high-performance liquid chromatography
L-Asn	L form asparagine
L-Gln	L form glutamine
L-Pro	L form proline
L-Ser	L form serine
p	probability
R^2	amount of reduction in the variability of Y obtained by using the regressor variable x_1, x_2. . . . in the model
RSM	response surface methodology
SMF	submerged fermentation
SSF	solid-state fermentation
WB	wheat bran
x_i, x_j	coded independent variables
X_i	natural variable of the factor
X_0	value of the natural variable at the center point
ΔX_i	the step change value
Y	predicted response

Greek symbols

α	the distance of the star points from the centre-point
β_0, β_i,	constant coefficients
β_0, β_i, β_{ii}, β_{ij}	constant coefficients

Iturin A is a cyclic lipopeptide containing a heptapeptide (L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser) cyclised with a β-amino fatty acid, it is a small molecules yet displays strong antifungal activity. In contrast, the other lipopeptide, surfactin which is a biosurfactant constituted by heptapeptide cyclised with a β-hydroxy fatty acid, has weak antibiotic activity. The strong efficacy of iturin A against various phytopathogenic fungi is similar to the available chemical pesticides (Phae and Shoda, 1990; Phae *et al*., 1990). Along with its wide spectrum of antibiotic activity and surface activity, iturin A confers low toxicity, low allergic effect on human and animals (Delcambe *et al*., 1977), high biodegradability; these characteristics qualify itself as a candidate for environmentally safe biological pesticide (Phae *et al*., 1992).

Thus far, most microbial cultivation has been conducted in submerged fermentation (SMF). However, many investigators have turned their attention to the solid-sate fermentation owing to the fact that solid-state fermentation (SSF) offers multitude advantages over sub-merged fermentation in that it requires less energy for cultivation because it does not need any agitation unit, it can be used for treatment of solid waste and it uses less solvent for product extraction (Adams *et al*., 2002; Holker and Lenz, 2005; Robinson *et al*., 2001). The use of *B. subtilis* to produce anti-fungal peptide antibiotic-iturin A and surfactin in solid-state fermentation (Akihiro *et al*., 1993; Mizumoto *et al*., 2006; Ohno *et al*., 1992, 1995, 1996) has been studied and reported; it was often reported that the amount of iturin produced per unit weight of wet substrate was five to ten times more than that in the sub-merged fermentation (Mizumoto *et al*., 2006; Ohno *et al*., 1992).

Previous works on iturin fermentation were mostly conducted using ‘‘one-factor-at-a-time-technique’’. Unfortunately, it frequently fails to locate the region of optimum response because the joint effects of factors on the response are not taken into account in such procedure. It was reported that the complexities and uncertainties associated the large-scale fungi fermentation usually come from lack of knowledge of the sophisticated interactions among various factors. The response surface methodology (RSM) has been increasingly used for various phases of an optimization process in fermentation (Buchanan and Philips, 1990; Haltrich *et al*., 1993; Prapulla *et al*., 1992; Shih *et al*., 2002; Shih and Shen, 2006). It is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such a technique (Box and Wilson, 1951). Recently, response surface methodology has been adopted to optimize the medium components of *B. subtilis* MO-01 for cyclic lipopeptide production in shake flask fermentation, which confirmed RSM is qualified to optimize the lipopeptide culture medium (Gu *et al*., 2005).

One of the objectives of biotechnology is the utilization of agricultural and food industry wastes for production of energy, chemicals, and protein animal feed, and at the same time combating pollution of the environment. Approximately 4.5 million tons of rice bran is produced in the world per year and their proper utilization has not been fully explored except for them to be used as stock feed. The effective utilization of such agricultural waste not only solves environmental problems, but also promotes the economic value of the agricultural products. These attractive characteristics further enhance the values of developing *B. subtilis* S3 as bio-control agent. Using rice bran in the present study, we adopt RSM to optimize the cultivation conditions of *B. subtilis* S3 for the enhancement of iturin A production in solid-state fermentation.

2. Materials and methods

2.1. Chemicals, microorganism and seed culture

Reagents for cultivation such as high gluten flour (containing 12.5% of protein), rice bran, peanut oil (product of Ta-Tung Co., Taiwan) were obtain from local supermarket. D-Glucose, K_2HPO_4, KH_2PO_4, $FeSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$ were obtained from Katamaya Co., Japan. The nutrient agar and Luria Bertani broth were obtained from DIFCO Laboratories Michigan, USA. All other reagents used were of the highest grade available unless otherwise indicated. *B. subtilis* S3, isolated from soil, was obtained from Taiwan Agricultural Research Institute, Wufeng, Taiwan. The strain was maintained on nutrient agar slants. Unless otherwise mentioned, the slant was incubated at 30 °C and then stored at 4 °C, which was subcultured every 4 weeks.