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Compactin Production Studies Using *Penicillium brevicompactum* Under Solid-State Fermentation Conditions

N. S. Shaligram · S. K. Singh · R. S. Singhal · A. Pandey · G. Szakacs

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Abstract In the present study, compactin production by *Penicillium brevicompactum* WA 2315 was optimized using solid-state fermentation. The initial one factor at a time approach resulted in improved compactin production of 905 μg gds^{-1} compared to initial 450 μg gds^{-1}. Subsequently, nutritional, physiological, and biological parameters were screened using fractional factorial and Box–Behnken design. The fractional factorial design studied inoculum age, inoculum volume, pH, NaCl, NH_4NO_3, $MgSO_4$, and KH_2PO_4. All parameters were found to be significant except pH and KH_2PO_4. The Box–Behnken design studied inoculum volume, inoculum age, glycerol, and NH_4NO_3 at three different levels. Inoculum volume (p=0.0013) and glycerol (p=0.0001) were significant factors with greater effect on response. The interaction effects were not significant. The validation study using model-defined conditions resulted in an improved yield of 1,250 μg gds^{-1} compactin. Further improvement in yield was obtained using fed batch mode of carbon supplementation. The feeding of glycerol (20% *v*/*v*) on day 3 resulted in further improved compactin yield of 1,406 μg gds^{-1}. The present study demonstrates that agro-industrial residues can be successfully used for compactin production, and statistical experiment designs provide an easy tool to improve the process conditions for secondary metabolite production.

N. S. Shaligram · R. S. Singhal
Food Engineering and Technology Department, Institute of Chemical Technology,
University of Mumbai, Matunga, Mumbai 400 019, India

S. K. Singh (✉) · A. Pandey
Biotechnology Division, National Institute for Interdisciplinary Science and Technology,
Trivandrum 695 019, India
e-mail: sudheer.vns@gmail.com

G. Szakacs
Department of Applied Biotechnology and Food Sciences,
Budapest University of Technology and Economics, Geller ter 4, 1111 Budapest, Hungary

Present address:
S. K. Singh
Microbiology Division, Central Drug Research Institute (CDRI), Chattar Manzil Palace, P.O. Box 173,
Lucknow 226 001, India

Keywords Compactin · *Penicillium brevicompactum* WA 2315 · Solid-state fermentation · Fractional factorial design · Box–Behnken design

Abbreviations

μg	Microgram
gds	Gram dry substrate
SSF	Solid-state fermentation
CoA	Coenzyme A
DiAHP	Di-ammonium hydrogen phosphate
ADiHP	Ammonium di-hydrogen phosphate
COC	Coconut oil cake
SOC	Sesame oil cake
GOC	Groundnut oil cake
PKOC	Palm kernel oil cake
OOC	Olive oil cake
WB	Wheat bran
RB	Rice bran
RH	Rice husk
SM	Soybean meal

Introduction

Compactin and its hydroxyl derivative, pravastatin, are potent inhibitors of cholesterol biosynthesis and are useful against atherosclerosis. It acts as competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl CoA and is prepared by microbial hydroxylation of compactin. Commercially, compactin is produced by *Penicillium citrinum* [1–4], *Penicillium cyclopium* [5], and *Aspergillus terreus* [6]. Improvement in the yield of compactin has been attempted by mutagenesis [1, 3]. Placket–Burman and central composite design has been reported by Chakravarti and Sahai [7] for the nutritional and environmental conditions for submerged culture of mutated *P. citrinum* NCIM 768 for compactin production. Compactin production by solid-state fermentation (SSF) was reported by Biocon, India [8].

The fermentation process development involves screening of large number of nutritional, biological, and physiological parameters. The screening by traditional method of one factor at a time approach results in performing a large number of experiments over an extended period of time. The statistical designs such as fractional factorial designs are a subset of full factorial design, which can be used to screen large number of factors with in a limited time period with substantial reduction in time and cost and help in identifying the important process parameters, while Box–Behnken designs are similar to central composite designs, but do not consider the extreme points [9]. The solid-state fermentation process offers major advantage in the form of use of cost-effective agro-industrial residues as substrate and uses simple instrumentation and less processing of fermented material during downstream processing. The SSF process is being increasingly explored for production of industrially important microbial enzymes and secondary metabolites [10–12]. Oil cakes have been utilized as raw materials for the fermentative production of various enzymes and biomolecules [13].

In our earlier study, we have reported production of compactin under SSF conditions using *Penicillium brevicompactum* WA 2315 and wheat bran + groundnut oil cake as carbon and support matrix. However, yield was improved up to 815 μg gds^{-1} only [11]. The optimized levels obtained by L_{25} orthogonal array were as follows: glucose 11% (*w*/*v*), maltose 5% (*w*/*v*),

glycerol 16% (*w*/*v*), di-ammonium hydrogen phosphate (DiAHP) 2.3% (*w*/*v*), $MgSO_4$ 0.75% (*w*/*v*), and KH_2PO_4 2.0% (*w*/*v*). The experiments were carried out at 25±2 °C for 7 days. Initial pH during the study was 6.5±0.2 with initial moisture content of 58%. Further optimization of pH gave maximum production of compactin at pH 7.5. In an effort to improve the yields further, minimal salt supplementations were studied with various agro-industrial residues. It was observed that under minimal salt supplementation conditions, soybean meal was a better carbon source and support matrix than wheat bran (WB) and groundnut oil cake (GOC). In the present study, we report on screening of factors to find the fermentation conditions with soybean meal as a support and carbon and nitrogen source.

Materials and Methods

Media Components

Glucose, glycerol, sucrose, maltose, lactose, fructose, magnesium sulfate, ammonium chloride, ammonium sulfate, yeast extract, casein peptone, and mycological peptone were purchased from Hi-Media Limited, Mumbai, India. Potassium di-hydrogen phosphate, sodium nitrate, potassium nitrate, ammonium nitrate, sodium chloride, and acetonitrile HPLC grade were purchased from S.D. Fine chemicals Limited, Mumbai, India. Urea, DiAHP, and ammonium di-hydrogen phosphate (ADiHP) were purchased from Merck Ltd, Mumbai, India. Corn steep liquor (CSL) was provided by Lumis Biotech Ltd, Mumbai. Maltodextrin was provided by Casco Inc. Canada. All the substrates for SSF were purchased from local market, Trivandrum, India. Rice bran and rice husk were procured from local market, Mumbai.

Microorganism Maintenance and Seed Preparation

P. brevicompactum WA 2315 used in the present study was obtained from the culture collection of Technical University of Budapest, Hungary. The culture was maintained on potato dextrose agar slants at 25 °C for 12 days; the slants were sub-cultured every month. A spore suspension (10^8 mL^{-1}) prepared from such slants was used to inoculate 50 mL of sterile seed medium in 250-mL flasks at 25 °C, 180 rpm for 2 days in incubator shaker. Seed medium contained (per liter): glucose (20 g), glycerol (30 g), peptone (8 g), $MgSO_4$ (1 g), $NaNO_3$ (2 g), and soybean meal (20 g), and the pH was adjusted to 6.5±0.2.

Fermentation

Supplement was added to 5 g substrate taken in a conical flask so as to adjust the initial moisture content to 58%. Supplement contained (percent *w*/*v*) ammonium nitrate 0.5, KH_2PO_4 0.2, $MgSO_4$ 0.1, and NaCl 0.1 [14], and pH was adjusted to 6.5±0.2 with 2 N phosphoric acid. All the media components were sterilized at 121 °C for 15 min. Fermentation was carried out at 25±2 °C for 7 days. Seed culture (2 mL) was added per 5 g of substrate. All the experiments were done in triplicates. Initially, 2 mL of 48-h-old seed culture was used to inoculate the individual flasks.

Selection of the Substrate

Various oil cakes such as coconut oil cake (COC), sesame oil cake, GOC, palm kernel oil cake, olive oil cake (OOC), and traditional agro-industrial residues such as WB,

rice bran, rice husk, and soybean meal (SM) were screened as substrates for compactin production. Elemental analysis of the substrate (SM) was performed (FLASH EA 1112 Series, Thermofinnigan, Italy) at the Indian Institute of Technology (IIT) Mumbai, India.

Effect of Initial Moisture Content

To investigate the influence of the initial total moisture content of the substrate, fermentation was carried out at various initial moisture contents (44%, 50%, 58%, 64%, and 70%) with SM and glycerol as an additional carbon source (22% *v*/*v*).

Effect of Seed Culture Growth Period

To study the effect of seed culture growth period on compactin production, seed media were incubated for 24, 48, 72, and 96 h. From the seed culture, 2 mL was inoculated into the production media.

Fermentation Time

To study the effect of fermentation time on maximum production of compactin, flasks were removed at different time intervals of 144, 168, and 192 h of fermentation and were analyzed for compactin production.

Effect of Additional Carbon and Nitrogen Sources

To study the effect of additional carbon sources on production of compactin, glycerol in the supplement was replaced with different carbon sources such as fructose, lactose, glucose, sucrose, maltose at 0.05 M, and maltodextrin at 0.1%.

The effect of nitrogen on compactin production was studied by replacing ammonium nitrate with different organic and inorganic nitrogen sources. The inorganic nitrogen sources selected for this study involved potassium nitrate, ammonium sulfate, ammonium di-hydrogen phosphate, di-ammonium hydrogen phosphate, ammonium nitrate, and ammonium chloride. The inorganic nitrogen sources were incorporated at 0.1 M. The organic nitrogen sources selected for the study were casein peptone, yeast extract, mycological peptone, urea, and CSL. The organic nitrogen sources were incorporated at 0.8% *w*/*v*.

Effect of glycerol

To study the effect of initial glycerol concentration on production of compactin in SSF, glycerol was added with final concentration as 5.5%, 11%, 16.5%, 22%, and 33% *v*/*v*, respectively. The glycerol concentration of 22% *v*/*v* was used as control.

Effect of Initial pH of Supplement

In order to study the effect of pH on compactin production, fermentation experiments were carried out at different initial pH values of supplement solution. The pH was varied between pH 4.5 and pH 8.5.

Batch Study

In order to study the product and biomass formation profile along with glycerol consumption, a study was performed. The individual flasks were harvested at 24-h interval till day 8 and analyzed for respective parameters.

Fractional Factorial Design

Factorial designs are a type of design of experiments that allow researchers to study the interacting effects of various factors on process outputs. Fractional factorial designs are a subset of full factorial designs. A 2^{7-3} design with seven factors and comprising of total 16 runs was used to screen the main effects. The high and low levels selected for this study represented the extremes of normal operating ranges. DOE software package (Design-Expert software from Stat-Ease, Inc., www.statease.com) was used to calculate all effects that could be estimated from the data. The factors were inoculum age (*A*), inoculum volume (*B*), pH (*C*), NaCl (*D*), ammonium nitrate (*E*), $MgSO_4$ (*F*), and KH_2PO_4 (*G*). The experimental design is shown in Table 1.

Box–Behnken Design

Effect of inoculum volume, inoculum age, glycerol, and ammonium nitrate was further studied for the optimization studies using a response surface methodology (RSM). A Box–Behnken design with four variables at three levels and a total of 29 runs were used for the study. The three levels of each variable were coded as −1, 0, and +1, which corresponded to the lower, middle, and higher values, respectively. For individual parameters, these were inoculum volume (*A*), inoculum age (*B*), glycerol (*C*), and ammonium nitrate (*D*). The

Table 1 Fractional factorial experimental design and response of the factors.

Run no.	Inoculum age (h)	Inoculum volume (mL)	pH	NaCl (%)	Ammonium nitrate (%)	$MgSO_4$ (%)	KH_2PO_4 (%)	Compactin (μg gds^{-1})
1	48 (−1)	1 (−1)	7 (1)	0.1 (−1)	1.6 (1)	0.3 (1)	0.5 (1)	838±64
2	48 (−1)	1 (−1)	6 (−1)	0.1 (−1)	0.4 (−1)	0.1 (−1)	0.2 (−1)	880±69
3	48 (−1)	1 (−1)	6 (−1)	0.3 (1)	0.4 (−1)	0.3 (1)	0.5 (1)	829±31
4	72 (1)	2 (1)	7 (1)	0.3 (1)	1.6 (1)	0.3 (1)	0.5 (1)	393±35
5	48 (−1)	2 (1)	7 (1)	0.3 (1)	0.4 (−1)	0.3 (1)	0.2 (−1)	755±64
6	72 (1)	1 (−1)	7 (1)	0.3 (1)	0.4 (−1)	0.1 (−1)	0.5 (1)	453±23
7	72 (1)	2 (1)	6 (−1)	0.1 (−1)	0.4 (−1)	0.3 (1)	0.5 (1)	394±25
8	72 (1)	2 (1)	6 (−1)	0.3 (1)	0.4 (−1)	0.1 (−1)	0.2 (−1)	395±11
9	72 (1)	1 (−1)	6 (−1)	0.1 (−1)	1.6 (1)	0.1 (−1)	0.5 (1)	524±35
10	72 (1)	1 (−1)	7 (1)	0.1 (−1)	0.4 (−1)	0.3 (1)	0.2 (−1)	577±49
11	48 (−1)	2 (1)	6 (−1)	0.3 (1)	1.6 (1)	0.1 (−1)	0.5 (1)	585±57
12	48 (−1)	1 (−1)	7 (1)	0.3 (1)	1.6 (1)	0.1 (−1)	0.2 (−1)	851±65
13	48 (−1)	2 (1)	7 (1)	0.1 (−1)	0.4 (−1)	0.1 (−1)	0.5 (1)	537±47
14	48 (−1)	2 (1)	6 (−1)	0.1 (−1)	1.6 (1)	0.3 (1)	0.2 (−1)	523±6
15	72 (1)	2 (1)	7 (1)	0.1 (−1)	1.6 (1)	0.1 (−1)	0.2 (−1)	235±27
16	72 (1)	1 (−1)	6 (−1)	0.3 (1)	1.6 (1)	0.3 (1)	0.2 (−1)	507±52

Results are mean ± SD of three determinations. Values in parentheses are coded values

software Design-Expert (Version 6.0.6, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis, and quadratic model building. The response surface graphs were obtained using the software to understand the effect of variables individually and in combination and to determine their optimum levels. The experimental setup of RSM is shown in Table 3.

Fed Batch Study

To study the effect of intermittent feeding of additional carbon source on compactin production, 0.5 mL of glycerol (10%, 20%, 40%, and 60% *v*/*v*) was added from days 2 to 5 in individual flasks at defined time interval of 24 h and mixed with the fermentation media by gentle shaking. The addition of glycerol started with 48 h onward, while in control flask, glycerol was added initially and no subsequent addition was done.

Analytical Method

Compactin from the fermented substrate was estimated by HPLC using the procedure as described by Konya et al. [4]. The fermented substrate was extracted with 50 mL ethyl alcohol at 25±2 °C on an orbital shaker at 180 rpm for 1 h. The extract was then centrifuged at 6,000 rpm using a cold centrifuge (Remi compufuge CPR-30, Mumbai, India). The supernatant was injected onto HPLC. Jasko HPLC system fitted with a reverse phase column Waters Spherisorb 5 μ ODS2 (4.6 mm×250 mm) was used. The mobile phase consisted of acetonitrile/water (60:40) adjusted to pH 3±0.2 by H_3PO_4. The flow rate was maintained at 0.8 mL min^{-1}, and the detection was done at 237 nm. Compactin from Themis Medicare, Mumbai was used as the standard.

Biomass Measurement

Fungal biomass estimation was carried out by determining the *N*-acetyl glucosamine released by the acid hydrolysis of the chitin, present in the cell wall of the fungi [15]. Fermented matter (0.5 g, dry weight basis) was mixed with concentrated sulfuric acid (2 mL), and the reaction mixture was kept for 24 h at room temperature (30 °C). This mixture was diluted with distilled water to make 1 N solution, autoclaved (15 psi for 1 h), neutralized with 1 N NaOH, and made to 100 mL with distilled water. The solution (1 mL) was mixed with 1 mL acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 mL) was added followed by the addition of 1 mL Ehrlich reagent and incubated at 65 °C for 10 min. The optical density of the reaction mixture was read at 530 nm against the reagent blank after cooling [16]. Glucosamine (Sigma) was used as the standard. The results were expressed as milligrams glucosamine per gram dry substrate (gds).

Results

Substrate Screening

Screening of various oil cakes along with wheat bran, rice bran, and rice husk showed maximum compactin production of 450 μg $gds^{-1}$with soybean meal (100%) as carbon source and support matrix followed by 407 μg gds^{-1}on COC (90%). The actual and

relative yield of compactin on other carbon substrates were as provided: 377 μg gds^{-1} (84%) on wheat bran, 278 μg gds^{-1} (62%) on rice bran, 255 μg gds^{-1} (57%) on palm kernel oil cake, 212 μg gds^{-1} (47%) on rice husk, 205 μg gds^{-1} (46%) on groundnut oil cake, 195 μg gds^{-1} (43%) on sesame oil cake with minimum production of compactin obtained from OOC being 143 μg gds^{-1} (32%). The elemental analysis of the substrate was performed (IIT-Mumbai, India). Soybean meal contained 43.30% carbon and 8.5% nitrogen content. The soybean meal was chosen as the substrate for further experimentation.

Effect of Moisture Content, Incubation Time, and Fermentation Time

Initial moisture (50%) was found to be the optimum with maximum compactin production of 900 μg gds^{-1}. The decrease in compactin yield was observed at initial moisture contents of 58% (840 μg gds^{-1}), 64% (822 μg gds^{-1}), 70% (507 μg gds^{-1}), and 44% (656 μg gds^{-1}), respectively. The seed culture with 48 h growth supported maximum production of 870 μg gds^{-1}, with decreased compactin yield being observed at 24 h (584 μg gds^{-1}), 72 h (836 μg gds^{-1}), and 96 h (827 μg gds^{-1}). The seed culture showed exponential growth phase from 24 to 96 h with 24 h being early log phase, while in 48–72 h, it was in mid-log phase. The seed culture growth in 72–96 h was of late log phase with 96 h showing early stationary phase as the biomass growth profile started flattening. The *N*-acetyl glucosamine content of culture was 2.25, 4.5, 6.0, and 6.25 mg mL^{-1} at 24, 48, 72, and 96 h of growth, respectively. The optimum time of compactin production was found to be 168 h with a compactin yield of 890 μg gds^{-1}. A decreased compactin yield was observed at 192 h (840 μg gds^{-1}) and 144 h (820 μg gds^{-1}).

Effect of Carbon and Nitrogen Supplements

Effect of supplementation of different carbohydrates such as sucrose, fructose, lactose, glucose, maltose, and maltodextrin were studied by substituting glycerol as additional carbon source. Glycerol supported maximum production of 860 μg gds^{-1} of compactin (Fig. 1). Figure 2 shows the effect of additional organic and inorganic nitrogen sources on compactin production, respectively. Ammonium nitrate of control was replaced with organic nitrogen sources and inorganic nitrogen sources. Since maximum yield (874 μg gds^{-1}) was observed in control flask with ammonium nitrate, hence, it was retained as additional nitrogen supplement during further studies.

Effect of Glycerol

To study the level of initial glycerol required for improved production of compactin, different concentrations of glycerol were studied. It was observed that an initial glycerol concentration of 22% (*v*/*v*) resulted in the maximum (877 μg gds^{-1}) compactin production (Fig. 3). Further increase in glycerol concentration decreased the production of compactin.

Effect of Initial pH on Compactin Production

An initial pH of 6.5 was found to be optimum (Fig. 4) with maximum production of 879 μg gds^{-1} of compactin being observed at this pH. A further increase in pH decreased the compactin production. The study with different parameters suggested that one factor at a time approach had reached to a stable production zone, and further increase in

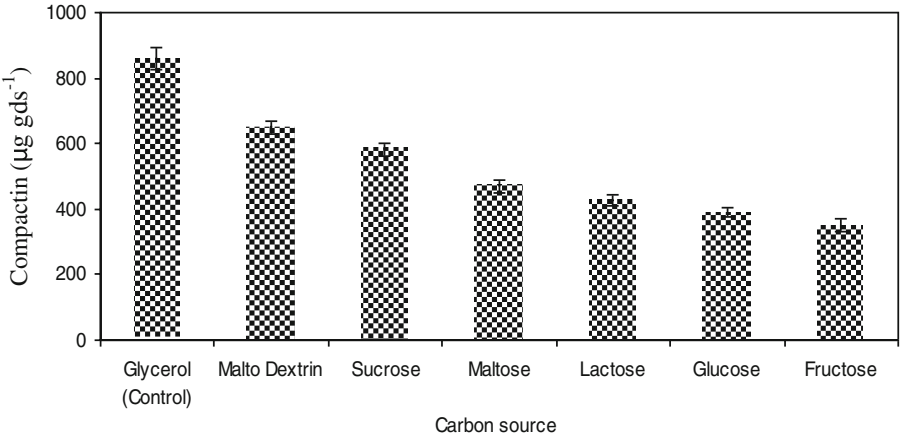

Fig. 1 Effect of carbon supplementation on compactin production by *P. brevicompactum* WA 2315 under SSF

production was not observed. Hence, to further improve the production, statistically designed experiments were used to identify the optimum factor levels with maximum effect of yield.

Batch Study

The data from Fig. 5 indicate that the product formation started from day 3, during which the glycerol was rapidly utilized. This suggests that the initial utilization of carbon source was mainly to build up the biomass for the growth. The production profiling of compactin suggested that production started on day 3 and continued to increase up to day 7, after which a decrease in production was seen on day 8. On day 7, the production of compactin was 905 µg gds^{-1}. Also, decrease in biomass was also observed after day 7.

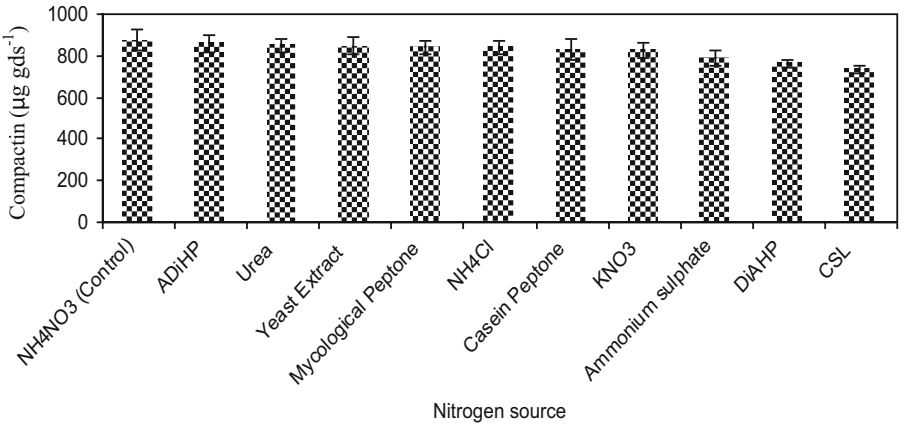

Fig. 2 Effect of nitrogen supplementation on compactin production by *P. brevicompactum* WA 2315 under SSF. The abbreviations DiAHP and ADiHP refer to $(NH_4)_2HPO_4$ and $(NH_4)H_2PO_4$, respectively

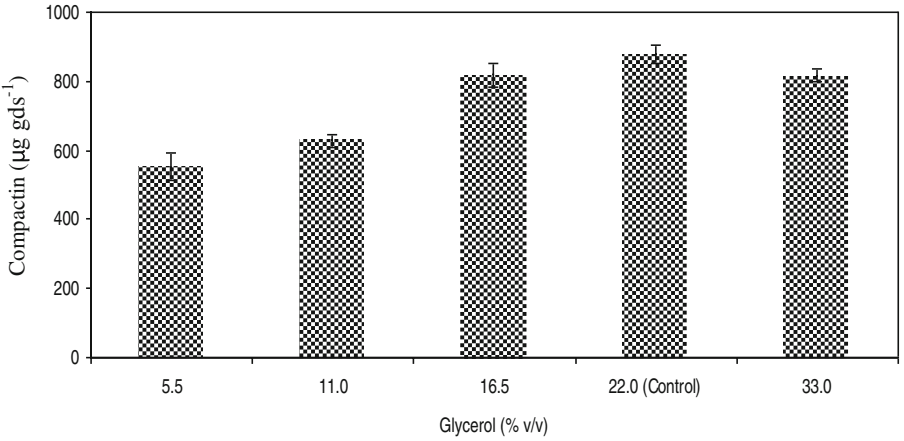

Fig. 3 Effect of glycerol on compactin production by *P. brevicompactum* WA 2315 under SSF

Fractional Factorial Design

The fractional factorial design with seven factors consisting of total 16 runs in one block (2^{7-3}) of resolution IV was used to screen the main effects. The two and higher-order interaction effects were confounded. The analysis of variance (ANOVA) of design suggested that out of seven factors screened, inoculum age, inoculum volume, NaCl, ammonium nitrate, and $MgSO_4$ had a significant effect on compactin production (Table 2). The effect of factors was observed to be both positive and negative, with inoculum age, inoculum volume, NH_4NO_3, and KH_2PO_4 being negative contributors to response, while the pH, NaCl, and $MgSO_4$ were positive contributor to response. However, only significant factors with major contribution such as inoculum age, inoculum volume, and NH_4NO_3 along with glycerol were selected for further optimization by Box–Behnken design. The positive and negative sign to response indicated that increasing or decreasing the

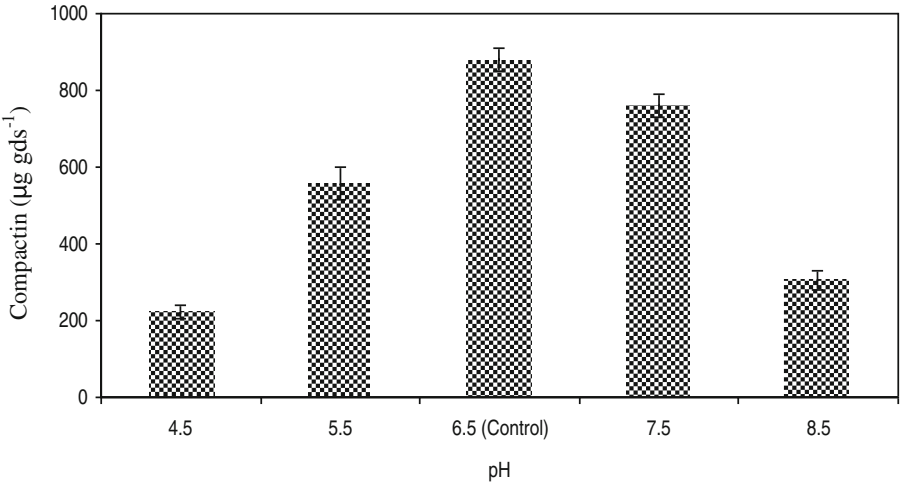

Fig. 4 Effect of initial pH on compactin production by *P. brevicompactum* WA 2315 under SSF

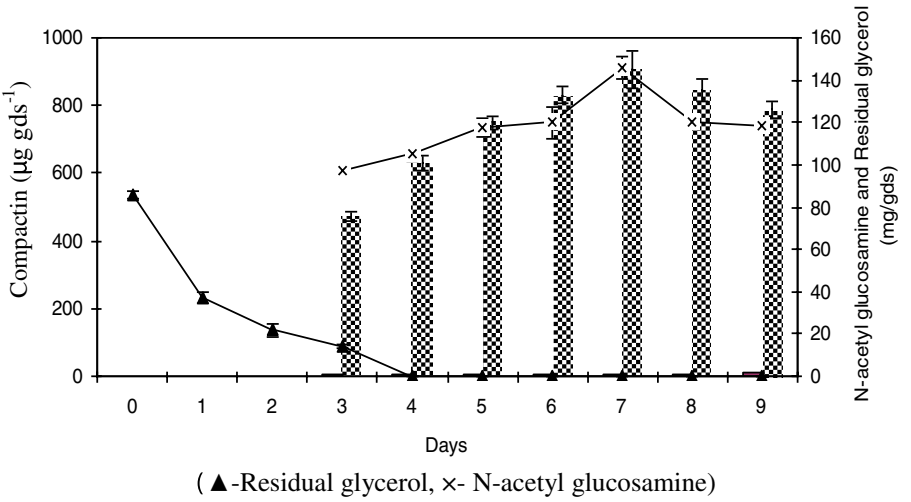

Fig. 5 Batch study on compactin production by *P. brevicompactum* WA 2315 under SSF

concentration of factors will lead to improved compactin production, and accordingly, levels of factors in Box–Behnken design were selected (Table 2).

Box–Behnken Design

The experimental runs and results for the Box–Behnken design are shown in Table 3. The maximum and minimum compactin yield value obtained in the design were 1,306 µg gds^{-1} (run no. 27) and 616 µg gds^{-1} (run no. 13), respectively (Table 3). Response surface optimization is more advantageous as compared to the traditional single parameter optimization. It saves time, space, and raw material. There were a total of 29 runs for optimizing the four individual parameters in the current Box–Behnken design. The current design was applied to the production of compactin. The data were analyzed by multiple regression analysis using the Design-Expert software, and the following polynomial Eq. 1 was derived to represent compactin yield as a function of the independent variables tested.

Table 2 Analysis of variance (ANOVA) for the fractional factorial design showing factors and their effect on response.

Source	Sum of squares	Coefficient estimate	Standard error	Mean square	*F* value	*p* value
Inoculum age (*A*)	336,400	−145	1.12	336,400	16,612.4	0.0049
Inoculum volume (*B*)	168,510	−102.62	1.12	168,510	8,321.49	0.007
pH (*C*)	0.25	0.12	1.12	0.25	0.01	0.9296
NaCl (*D*)	4,225	16.25	1.12	4,225	208.64	0.044
NH_4NO_3 (*E*)	8,281	−22.75	1.12	8,281	408.93	0.0315
$MgSO_4$ (*F*)	7,921	22.25	1.12	7,921	391.16	0.0322
KH_2PO_4 (*G*)	1,806.25	−10.62	1.12	1,806.25	89.19	0.0672

Table 3 Box–Behnken design and response of the factors.

Run	Inoculum volume (mL)	Inoculum age (h)	Glycerol (% *v*/*v*)	NH_4NO_3 (% *w*/*v*)	Compactin (μg gds^{-1})
1	0 (1.5)	1 (72)	−1 (8)	0 (0.8)	851±41
2	0 (1.5)	0 (60)	0 (14)	0 (0.8)	1,136±55
3	0 (1.5)	1 (72)	1 (20)	0 (0.8)	1,273±61
4	−1 (1.0)	0 (60)	0 (14)	−1 (0.4)	998±45
5	−1 (1.0)	0 (60)	1 (20)	0 (0.8)	1,075±56
6	1 (2.0)	−1 (48)	0 (14)	0 (0.8)	884±43
7	1 (2.0)	1 (72)	0 (14)	0 (0.8)	1,047±58
8	0 (1.5)	0 (60)	−1 (8)	−1 (0.4)	665±36
9	0 (1.5)	1 (72)	0 (14)	−1 (0.4)	1,088±59
10	0 (1.5)	0 (60)	0 (14)	0 (0.8)	1,136±55
11	0 (1.5)	0 (60)	0 (14)	0 (0.8)	1,136±55
12	0 (1.5)	1 (72)	0 (14)	1 (1.2)	926±55
13	1 (2.0)	0 (60)	−1 (8)	0 (0.8)	616±35
14	0 (1.5)	−1 (48)	0 (14)	1 (1.2)	978±57
15	0 (1.5)	0 (60)	0 (14)	0 (0.8)	1,136±55
16	−1 (1.0)	−1 (48)	0 (14)	0 (0.8)	1,170±78
17	0 (1.5)	0 (60)	1 (20)	−1 (0.4)	1,070±63
18	0 (1.5)	−1 (48)	−1 (8)	0 (0.8)	943±51
19	−1 (1.0)	0 (60)	−1 (8)	0 (0.8)	745±38
20	0 (1.5)	−1 (48)	1 (20)	0 (0.8)	1,157±70
21	0 (1.5)	0 (60)	−1 (8)	1 (1.2)	796±42
22	1 (2.0)	0 (60)	1 (20)	0 (0.8)	912±48
23	0 (1.5)	0 (60)	0 (14)	0 (0.8)	1,136±55
24	−1 (1.0)	0 (60)	0 (14)	1 (1.2)	1,009±65
25	0 (1.5)	−1 (48)	0 (14)	−1 (0.4)	1,087±37
26	1 (2.0)	0 (60)	0 (14)	1 (1.2)	716±35
27	−1 (1.0)	1 (72)	0 (14)	0 (0.8)	1,306±74
28	1 (2.0)	0 (60)	0 (14)	−1 (0.4)	770±47
29	0 (1.5)	0 (60)	1 (20)	1 (1.2)	1,305±65

Results are mean ± SD of three determinations. Values in parentheses are real values

The experimental data were statistically analyzed using the ANOVA and the results are shown in Table 4.

$$\begin{aligned}\text{Response} = {} & 1,136 - 113.16A + 22.66B + 181.33C + 4.33D - 136.45A^2 + 46.19B^2 \\ & - 116.45C^2 - 116.45D^2 + 6.75AB - 8.5AC - 16.25AD + 52BC \\ & - 13.25BD + 26CD \end{aligned} \quad (1)$$

The ANOVA of the quadratic regression model indicated that the model was highly significant with $p=0.0007$ and F value for the model being 6.31. There was only a 0.07% chance that a "Model F-Value" this large could occur due to noise. The coefficient estimate and the corresponding Prob > F values suggested that inoculum volume and glycerol had a significant effect on compactin production. From the F value statistic (Table 4), it was concluded that a change in glycerol concentration caused a major variation in compactin production. The R^2 statistic indicated that the model as fitted explained 86% of the

Table 4 Analysis of variance (ANOVA) for the Box–Behnken design showing factors and their effect on response.

Source	Sum of squares	Coefficient estimate	Standard error	Mean square	*F* value	*p* value
Model	852,729.7	1,136	43.90	60,909.26	6.31	0.0007
Inoculum volume (*A*)	153,680.3	−113.16	28.34	153,680.33	15.94	0.0013
Inoculum age (*B*)	6,165.333	22.66	28.34	6,165.33	0.63	0.4372
Glycerol (*C*)	394,581.3	181.33	28.34	394,581.33	40.93	0.0001
NH_4NO_3 (*D*)	225.3333	4.33	28.34	225.33	0.02	0.8807
A^2	120,784.1	−136.45	38.55	120,784.07	12.52	0.0033
B^2	13,900.01	46.29	38.55	13,900.01	1.44	0.2497
C^2	87,973.25	−116.45	38.55	87,973.25	9.12	0.0092
D^2	87,973.25	−116.45	38.55	87,973.25	9.12	0.0092
AB	182.25	6.75	49.09	182.25	0.01	0.8926
AC	289	−8.5	49.09	289	0.02	0.8650
AD	1,056.25	−16.25	49.09	1,056.25	0.10	0.7455
BC	10,816	52	49.09	10,816	1.12	0.3074
BD	702.25	−13.25	49.09	702.25	0.07	0.7912
CD	2,704	26	49.09	2,704	0.28	0.6047

variability in compactin production. The adjusted R^2 value of 0.72 indicated the model to be significant. A very high degree of precision and a good degree of reliability of the experimental values were indicated by a low value of the coefficient of variation (CV= 9.79). The response surface plots were developed to study the interaction of significant effects to identify a robust response zone (Fig. 6). The optimum values for variables

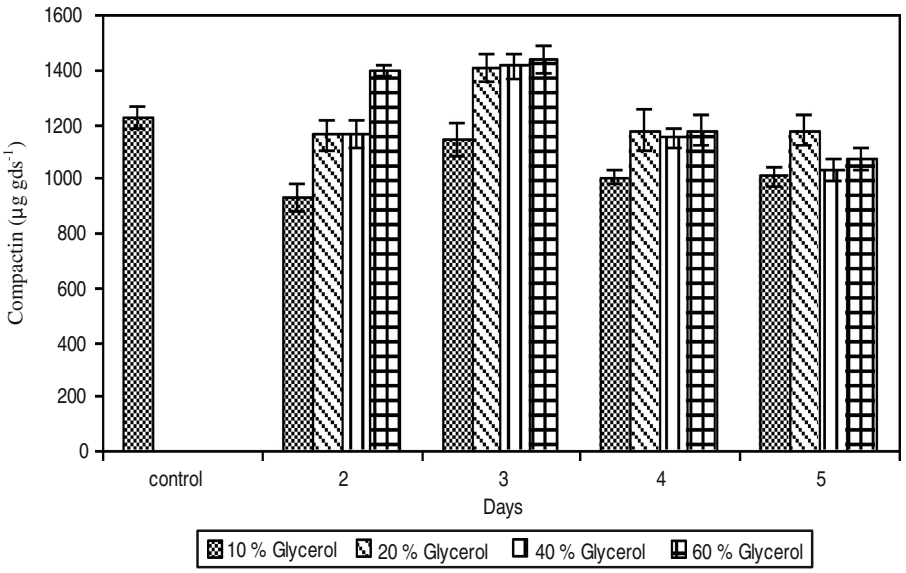

Fig. 6 Effect of fed batch on compactin production by *P. brevicompactum* WA 2315 under SSF

obtained were inoculum volume 1.5 mL, inoculum age 72 h, glycerol 20% *v*/*v*, ammonium nitrate 0.8% *w*/*v*, NaCl 0.16% *w*/*v*, $MgSO_4$ 0.16% *w*/*v*, and KH_2PO_4 0.32% *w*/*v*. A validation study based on model suggested optimum values resulted in a compactin yield of 1,250 μg gds^{-1} as compared with the model predicted yield of 1,310 μg gds^{-1}. This suggested that model predicted response zone was the best region for compactin production under defined experimental space.

Fed Batch Study

From the batch study, it was observed that the glycerol was utilized almost completely on day 4. Hence, glycerol was added to the flasks from days 2 to 5. Figure 7 demonstrates that the glycerol supplementation of 20% (*v*/*v*) on day 3 to the production flask further improved the compactin yield (1,406 μg gds^{-1}) as compared to the production obtained after Box–Behnken design since the yields obtained from 20% and 40% glycerol addition on day 2 were less than that from 60% addition and yields obtained on day 3 were almost comparable. Hence, day 3 addition of 20% glycerol was assumed to be the ideal for improved compactin yield.

Discussion

Filamentous fungi under natural conditions grow on solid supports, which act as a support matrix and nutrient source. The organisms gradually hydrolyze the support matrix and use the available nutrient for its growth. The solid-state fermentation tries to mimic the natural growth conditions for the organism by using nutritionally rich agro-industrial residues; such materials can also double up as support for fungal growth and in the process provide valuable byproducts. The selection of a substrate for solid-state fermentation process depends mainly on the cost and availability and thus may involve screening of several agro-industrial residues. The substrate that provides all the required nutrients to the microorganism could be considered as an ideal substrate [17]. Elemental analysis of soybean meal suggested that soybean meal was rich in carbon and nitrogen; however, the carbon may be of complex type. Hence, to ensure the availability of other limiting nutrients along with carbon and nitrogen during initial growth phase, additional supplementation in the form of minimal salt solution and simple carbon and nitrogen was necessary.

Substrate moisture (water activity, a_w) plays an important role in SSF. The moisture content plays an important role in solid-state fermentation, although fermentation with relatively no moisture to very high initial moisture levels (30–80% *w*/*w*) are reported [18]. However, high moisture content leads to aggregation of substrate particles, poor aeration, and possible anaerobic conditions [19], while very low moisture content restricts the fungal growth [20]. Optimal moisture content depends on the nature of microorganism and the substrate being used. For lovastatin production, 60% initial moisture and 6 days of fermentation produced maximum yield by *Aspergillus flavipes* [12], while in the present study, initial moisture content of 50% was observed to be optimum.

The improved production in flasks supplemented with glycerol suggested preference of organism for glycerol over other carbon sources. Similarly, better production in ammonium nitrate supplemented flask suggested that simple nitrogen source was preferred for initial growth, while later nitrogen requirements were taken care by complex nitrogen present in soybean meal. This study clearly demonstrated that glycerol was rapidly utilized during

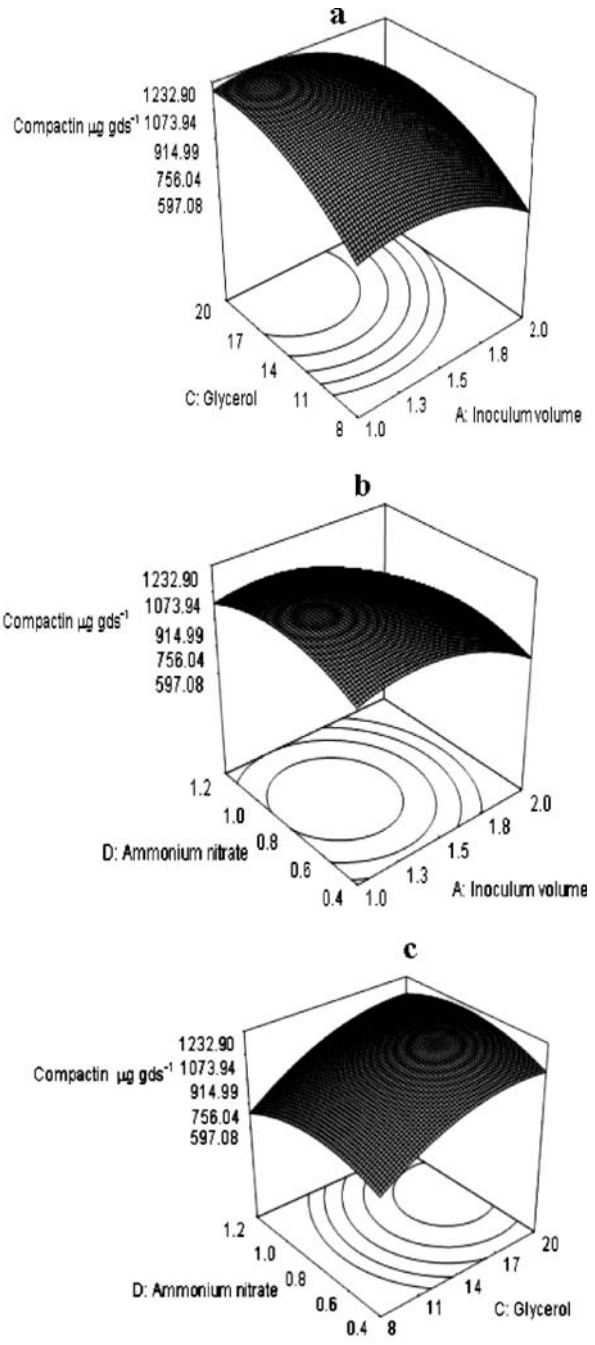

Fig. 7 Interaction graphs for Box–Behnken design. **a** Interaction plot between inoculum volume and glycerol. **b** Interaction plot between inoculum volume and ammonium nitrate. **c** Interaction plot between glycerol and ammonium nitrate

initial growth period, which was in contrast to earlier reports that suggested that glycerol was metabolized slowly during growth [5, 21].

The effect of pH studies in submerged fermentation for compactin production had been reported for *P. cyclopium*. An initial pH of the media maintained at 4.0 by 0.01 M citrate

buffer supported compactin production of 118 mg L^{-1} [5]. The process development studies for lovastatin production at Merck and Mevacor demonstrated pH control as one of the parameters leading to improved lovastatin productivity [21]. Similar effect of pH was observed on compactin production. In this study also, it was observed that within a controlled pH range of pH=6.8–8.0, compactin production was unaffected [22]. Although it was difficult to extrapolate submerged fermentation study with solid-state fermentation, the existing data on compactin production clearly suggested that within a controlled pH range, it can be improved.

From ANOVA of fractional factorial design, the positive and negative main effects were identified. The factors with major effects such as inoculum age, inoculum volume, and NH_4NO_3 were selected for further study based on their higher effect on response. The fractional factorial designs are basically screening designs, which had been successfully used for screening of main effects. Although, in the batch study, it was observed that compactin production started only after 72 h, which, being a secondary metabolite, was expected; however, most of initial glycerol was consumed during this period. Also, increase in biomass was observed till day 7 after which decrease in biomass and compactin production was observed. The glycerol had been preferably utilized for production of compactin [22] and clavulanic acid [23, 24]. In clavulanic acid biosynthesis, it had been observed that a glycerol concentration higher than 2% was inhibitory [25] under submerged fermentation conditions, which had not been the case in present study under SSF conditions. It was observed in the present study that good compactin yield was more dependent on the time of glycerol addition and less on concentration with 20%, 40%, and 60% glycerol addition resulting in almost comparable compactin yield when added on day 3, although the microbial systems used in both studies were different (*Streptomyces clavuligerus* and *P. brevicompactum*, respectively). However, from the present study, it can be assumed that microbial system was less prone to osmotic stress under SSF. The earlier studies for lovastatin production had also suggested that fungal physiology changes under SSF conditions and increased osmotolerance may be a result of this changed physiology [26]. Fed-batch fermentations had been extensively used for production of secondary metabolites [27–29], and in the present investigation also, it demonstrated successful improvement in the production.

In conclusion, the optimization of nutritional parameters suggested that an initial moisture content of 50% and 168 h of fermentation were optimum for compactin production. Supplementation with glycerol resulted in further improvements with glycerol profiling during the study, suggesting its rapid utilization during initial growth with complete utilization by 96 h. Compactin production was improved from 450 to 1,250 μg gds^{-1}, resulting in an improvement of 2.7 times using the combination of single factor and statistical experiment design. Fed-batch fermentation can be used for improved compactin production.

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