International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.5, No.3, pp 1393-1400, April-June 2013

IPACT-2013[14th – 15th March 2013]

National Conference on Industrial Pollution And Control Technology-2013

Optimization Of Compactin Production By Plackett-Burman Method Using Penicillium citrinum

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Abstract: Plackett-Burman design was used to optimize various media compositions of carbon and nitrogen sources along with other nutrient parameters influencing the production of compactin under submerge fermentation. Initially eight different carbon sources were tested individually for compactin production. Among them glucose containing medium had produce maximum compactin. Secondly eight different nitrogen sources were tested, in which the medium containing urea and soybean meal had produce maximum compactin. The above carbon and nitrogen source was chosen for further optimization, eight nutrients such as (glucose, glycerol, soya bean meal, KH_2PO_4, urea, PEG, $CaCl_3.H_2O$ and $MgSO_4.7H_2O$) were chosen for Plackett-Burman design with a 12 experimental runs. Among 8 factors studied by Plackett-Burman design, three factors had influenced the compactin production. The $MgSO_4.7H_2O$, KH_2PO_4 and glycerol medium constituents were found to be effective in the production of compactin by *Penicillium citrinum*. An improved compactin yield of 232.11 mg/L was obtained with optimized medium composition, which is higher production than previously obtained results.
Keywords: Compactin, *Penicillium citrinum*, Plackett-Burman design and submerge fermentation.

Introduction

Compactin a hypocholesterolemic molecule, is a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl (HMG)-Co A reductase, which is a regulatory enzyme for cholesterol biosynthesis and also acts as an antifungal agent. Compactin is also known as mevastatin and belongs to the polyketide group of metabolites. Endo et al[2] discovered three active compounds ML-236A, ML-236B and ML-236C in the culture broth of *P. citrinum*. At the same time, Brown et al[3] discovered an antifungal compound compactin in the culture broth of *Penicillium brevicompactum*. The structural similarity between the HMG- Co A and the δ-lactone ring of the acid form of statins plays an important role in the binding of compactin to the catalytic site of HMG-Co A reductase. This is supported by data showing that the inhibitory activity of compactin is reduced to 1/100 or less by acetylation of the hydroxy group at either C_3' or C_5, and 5′-phosphocompactin is ten times less active as an inhibitor than compactin itself [4]. Compactin acts by inhibiting the biosynthesis of mevalonic acid, which is a precursor of

steroidal and non-steroidal compounds, thereby having pleiotropic effects[6]. The first report on biosynthesis of compactin came in 1985[5]. The main skeleton of compactin, as well as lovastatin, is a polyketide of acetate origin. Statins are effective cholesterol lowering agents in humans. Cholesterol is an essential component of cell membranes and a precursor of various hormones, bile salts and vitamins. Cholesterol biosynthesis begins with an acetyl- Co A unit and takes more than 27 steps. Increased removal of LDL precursors resulting in decreased LDL production and decreased VLDL (very low-density lipoprotein) production make statins most effective agents for lowering LDL cholesterol, moderately effective for lowering triglycerides and mildly effective for raising HDL[7]. As Inhibition of HMG-Co A reductase results in accumulation of HMG-Co A that can be metabolised into simpler compounds, thereby making it an ideal target for treatment of atherosclerosis. Other molecules of this class include lovastatin, pravastatin, simvastatin, atorvastatin and fluvastatin. Some of these (e.g. compactin, lovastatin, pravastatin, simvastatin and fluvastatin) are structurally similar as they all have a hydroxy-hexahydro-naphthalene ring. Compactin and lovastatin are derived from fungal sources while pravastatin and simvastatin are chemical modifications of compactin and lovastatin, respectively.

Various hydroxylated and phosphorylated derivatives of compactin can be produced by growing specific microorganisms in culture medium containing compactin in addition to nutrients. Compactin is used as a substrate for microbial conversion into pravastatin, a widely used pharmaceutical drug for the treatment of hypercholesterolemia[8]. One more important derivative of compactin was known as ML-236A, discovered at the same time as compactin (ML-236B) by Endo et al[2] is less potent than compactin but serves as a precursor for a variety of semisynthetic compactin analogs and is therefore quite important commercially[12]. 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. Various fungi have been used for the commercial production of compactin. The different strategies have been used for improving production levels of compactin. Biochemical systems are multivariable process therefore, these systems are optimized in a number of steps. The first step is to screen important factors and the second to optimize those factors. One of the statistical designs for the screening of the independent variables is Plackett-Burman. It is a two factorial design and offers the screening of a large number of independent factors (N) in a small number of experiments (N+1). Factors chosen for study can be either the nutritional components or environmental conditions[1].

Materials and methods

Microorganisms and culture conditions

Penicillium citrinum MTCC 1751 was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained on potato dextrose agar slants at 4^{o} C and the slants were subcultured every month.

Media components

Potato dextrose agar (PDA), dextrose, galactose, mannose, sucrose, lactose, maltose, fructose, xylose, glycerol, peptone, soybean meal, yeast extract, malt extract, urea, ammonium chloride, ammonium sulphate, KH_2PO_4, $CaCl_3.H_2O$, polyethylene glycol (PEG) and $MgSO_4.7H_2O$ were purchased from Hi-Media Limited, India. HPLC grade acetonitrile and ethanol were purchased from Rankem, New Delhi, India. All the chemicals used were of analytical grade. Compactin standard was purchased from Sigma chemicals, Bangalore, India.

Inoculum preparation

Actively growing slants were used to prepare the spore suspension of *P. citrinum* in sterile water. 10% (v/v) spore suspension was inoculated into conical flasks containing the seed medium: 100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g $NH_4H_2PO_4$, 0.5 g $MgSO_4$.$7H_2O$ and 0.1 g $CaCl_2$ in 1000 mL of distilled water, adjusted to pH 6. These cultures were incubated at 30˚C for 48 h in a shaking incubator at 120 rpm. 5 percent of this preculture was used to inoculate into the production medium. Fermentation experiments were carried out at 30˚C for 10 days using *P. citrinum* in 250 mL Erlenmeyer flasks containing 100 mL of production media, as per the experimental design.

Extraction of compactin

After fermentation, the harvested samples were homogenized to recover the intracellular product. An equal volume of ethanol was added to fermentation broth and the suspension was kept in an incubated rotary shaker for 1 h at 200 rpm and 40˚C. The suspension was filtered through a whatman filter paper and then through a micro filter (Millipore) of 0.22 mm pore diameter. 20 µL of the filtrate was analyzed for compactin using HPLC.

Analysis of compactin

Analysis of compactin was carried out in Shimadzu HPLC (LC20 AT prominence) at 238nm in Luna C18 column of particle size 5m and (250X4.6) mm I.D, UV detector (SPD 20 A) and the column oven (CTO-10 AS vp) at 45°C. Binary gradient system was used and the samples were injected manually using Rheodyne injector of 20 µL. The mobile phase used was acetonitrile and 0.1 % orthophosphoric acid in the ratio of 60:40 respectively. The eluent was pumped at a flow rate of 1.5 mL/min. Compactin standard was obtained from Sigma-Aldrich and various concentrations of compactin were prepared by dissolving in acetonitrile. The equation of the standard curve for the various concentrations of compactin (Y) versus peak area (X) is Y= 49870 X with R^2 = 0.9952. The retention time of compactin elutes at 9.4 min of a fermented sample.

Plackett–Burman design

The PB design was proved to be a powerful tool to rapidly determine the effects of medium constituents on compactin production. In this part, the PB design was used to evaluate the relative importance of various nutrients for compactin production in batch fermentation. This design does not consider the interaction effects among the variables and is used to screen the important variables affecting the compactin production.

The experimental design for screening of medium components was shown in Tables 3 and 4. Each variable was set at two levels, that is, high level and low level. The high level of each variable was set far enough from the low level to identify which ingredients of the media have significant influence on the compactin production.

Results and discussion

Screening of carbon source

Initially various carbon sources have been tested for suitable growth of *P. citrinum* and maximum compactin production. Compactin is the secondary metabolite, the maximum growth of the organism is required and hence it depends on the type of carbon source. The various carbon sources used in our experimentation are glucose, fructose, galactose, mannose, sucrose, lactose, maltose and xylose. Among the above carbon sources only few had influence the growth of the organism and production of compactin, no nitrogen source was added. Glucose had produce maximum of compactin 106.6 mg/L and second highest was fructose 88.74 mg/L of compactin was shown in Table 1. We had chosen glucose for sole carbon source for further optimization experiments. High productivity is only possible in the presence of sufficient amounts of carbon source and additional precursors in the medium. The combination of glycerol and glucose had produced maximum amount of compactin 121.06 mg/L.

Lactose may be the most important factor influencing the production of mevinolinic acid. When lactose is depleted, the biosynthesis of mevinolinic acid is ceased. Lactose is both utilised for biomass formation and biosynthesis of mevinolinic acid. Therefore, the total lactose volumetric uptake rate is the sum of uptake rates for biomass growth and mevinolinic acid formation[9].

Table 1. Compactin production from various carbon sources

S.No	Carbon sources	Compactin Production mg/L
1	Glucose	106.6
2	Galactose	67
3	Fructose	88.74
4	Sucrose	56.34
5	Lactose	72.8
6	Maltose	37.7
7	Mannose	52.7
8	Xylose	71.8
9	Glycerol and glucose	121.06

Lactose has been considered as a possible alternative carbon source: to obtain a significant lovastatin yield. The conclusion was that high yield of lovastatin can be obtained in the presence of glycerol, glucose, and

reciprocal agitation, no matter which soybean flour is used. Alternatively, an intermediate yield of lovastatin can be obtained in the presence of glycerol, lactose, and defatted soybean flour, no matter which agitation used[10].

In our case, glucose was the best carbon source for highest compactin production and the combination of glucose and glycerol had produce highest compactin in individual screening of carbon sources.

Screening of nitrogen source

Nitrogen sources influence the production of compactin, hence screening of various nitrogen sources was carried out keeping glucose and other medium constituents constant. Various nitrogen sources used here are peptone, soybean meal, yeast extract, urea, ammonium chloride, ammonium sulphate and malt extract. Among the above nitrogen sources urea had high influence on compactin production and secondly soybean meal influence the production. When a nitrogen source was added there is sharp increase in the production of compactin was observed. The urea and glucose combination had produced maximum compactin of 169.78 mg/L secondly the soybean meal and glucose combination had produced 122.1 mg/L shown in Table 2.

As lovastatin does not contain any nitrogen moieties in its structure ($C_{24}H_{36}O_5$), its formation is connected with nitrogen utilization only to the extent to which the amount of nitrogen influences biomass amount in the system. Organic nitrogen does not directly participate in the biosynthesis of mevinolinic acid. Yeast extract is regarded as a sole nitrogen source as no ammonium salts are added to the medium[9].

Table 2. Compactin production from various nitrogen sources

S.No	Nitrogen source	Compactin production mg/L
1	Peptone	22.1
2	Soybean meal	122.1
3	Yeast extract	67.39
4	Urea	169.78
5	Ammonium chloride	100.64
6	Ammonium sulphate	23
7	Malt extract	12.9

A mutant strain of *P. citrinum* grown in a chemically defined production medium yielded compactin of 145 mg/L, while addition of a surfactant, Tween 80, increased compactin to 175 mg/L[11]. The significance between lactose/glycerol and glycerol/peptone were also important. Lovastatin production was more sensitivity the changes in lactose and peptone dosages[13]. Lactose and $(NH_4)_2SO_4$ significantly decreased lovastatin production at the concentrations used while corn steep liquor and soybean meal significantly enhanced lovastatin titres. $(NH_4)_2SO_4$ as a nitrogen source showed strong repression on lovastatin production. Plackett-Burman design, a 0.44 g/L of lovastatin titre in broth was measured in this procedure[13]. Production of compactin by *P. citrinum* was optimized using Placket-Burman design, out of 11 factors studied by Plackett-Burman design, six factors such as urea, glucose, harvesting time, glycerol, inoculum age and $MgSO_4$ influenced the compactin production[14].

Plackett-Burman experimental design was used to screen important nutrient parameters influencing the production of red pigments by *Monascus purpureus* MTCC 369 under submerged fermentation. Nine nutrient parameters were tested for PB experimental design among which, NH_4Cl, NaCl, KH_2PO_4, $MgSO_4.7H_2O$ and$MnSO_4.H_2O$ had contributed to a large extent, dextrose, $CaCl_2.2H_2O$ and $FeSO_4.7H_2O$ had little impact, while, $(NH_4)_2.SO_4$ contributes moderately for red pigment production by *M. purpureus* MTCC 369 under submerged fermentation[15]. In the first step of optimization, with Plackett–Burman design, soluble starch, urea and KH_2PO_4 were found to be the important factors affecting *Coniothyrium minitans* spore production significantly[16]. Effect of 19 different medium components on chitinase production by marine isolate *Pantoea dispersa* was studied by Plackett–Burman design, In the Plackett–Burman method, significant factors influencing the chitinase production were screened. 4.21-fold increase in chitinase production was observed in Plackett–Burman experimental design. Increase in production of endochitinase (3.95-fold) and chitobiase (2.31-fold) was observed[17].The production optimization of α-amylase (E.C.3.2.1.1.) from *Aspergillus niger* ATCC 16404 fungus is obtained with statistical experimental designs. This design has been chosen in order to select the factors favoring enzyme production[18]. Medium composition for transglutaminase (MTGase) production by *Bacillus circulans* BL32, a recently isolated strain from the Amazon basin,was optimized using a stepwise

strategy. Plackett–Burman (PB) statistical design was applied to find the key ingredients for the best medium composition among glycerol, sucrose, peptone, tryptone, Na_2HPO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$. Sucrose, negatively, and peptone, positively, have significant effects on MTGase production[19].

A statistical methodology, combining Plackett–Burman design with uniform design (UD), was applied to optimize the concentrations of different inorganic salt components in liquid fermentative medium for carotenoids production by *Rhodobacter sphaeroides*[20]. A screening approach, involving the use of the Plackett-Burman experimental design, permitted the evaluation of the effects of 25 parameters from the sample pretreatment stage (furnace heating temperature, furnace heating duration, cooling, filtration, addition of HCl) on the Pb, Cr and Al determination in macroalgae by graphite furnace atomic absorption spectrometry (GF-AAS)[21]. Xylanase was produced by *Aspergillus terreus* cultivated on finely ground wheat straw in solid-state fermentation. The optimal medium composition was developed by applying the Plackett-Burman experimental design[22]. A compactin producer *Penicillium* sp. strain was isolated from soil. The compactin biosynthesizing capacity of the strain was improved from 5 µg/mL to 250 µg/mL by mutation selection method. The effect of the medium composition on compactin productivity was investigated. A central, orthogonal three-factor experimental design by Box and Wilson was used for compactin production [23].

Media optimization using Plackett-Burman experimental design

Initially the carbon and nitrogen sources were screened and among them the best carbon and nitrogen sources were selected for further optimization using Plackett-Burman design. Plackett-Burman design was adopted to optimize various medium components for the production of compactin fermentation by *P. citrinum*. Various media components were investigated for their dominance in the process of compactin production. Table 3 shows the medium components for the independent variables and their respective high and low concentrations used in PB optimization study with respect to compactin production. Eight nutrients such as (glucose, glycerol, soya bean meal, KH_2PO_4, urea, PEG, $CaCl_3.H_2O$ and $MgSO_4.7H_2O$) were chosen for Plackett-Burman design with a 12 experimental runs was shown in Table 4. A factorial design, Plackett-Burman design was used to study the dominance among the eight constituents of the medium. The effects of the variables and their significance on the production were found using their P values ($P< 0.05$).

Table 3. Plackket-Burman design and media components compactin production by *P.citrinum*

Variables	Medium Components	Lower level (-1) g /100 mL	Higher level (+1) g / 100 mL
A	Glucose	5	7
B	Soya bean meal	3	5
C	Glycerol	0.4	0.5
D	KH_2PO_4	0.5	0.7
E	Urea	0.01	0.1
F	$CaCl_3.H_2O$	0.02	0.1
G	PEG	0.02	0.2
H	$MgSO_4.7H_2O$	0.01	0.2

Table 4. Represents placket-burman experimental design with 12 runs with corresponding compactin production.

Runs	A	B	C	D	E	F	G	H	Compactin mg/L
1	+	+	-	+	+	+	-	-	232.11
2	+	-	+	+	+	-	-	-	132.7
3	-	+	+	+	-	-	-	+	92.45
4	+	+	+	-	-	-	+	-	28.6
5	+	+	-	-	-	+	-	+	79
6	+	-	-	-	+	-	+	+	53.4
7	-	-	-	+	-	+	+	-	80.12
8	-	-	+	-	+	+	-	+	67.88
9	-	+	-	+	+	-	+	+	41.6
10	+	-	+	+	-	+	+	+	19.78
11	-	+	+	-	+	+	+	-	55.6
12	-	-	-	-	-	-	-	-	87.2

The effect of each variable was determined with the following equation.

$E_{xi} = 2 (\Sigma H_{xi} - \Sigma L_{xi}) / N$

Where, E_{xi} is the concentration effect of the tested variable, H_{xi} and L_{xi} are the concentration of compactin at high level and low level of the same variable, Among the variables tested, the variables which were found to be dominant on the production of compactin in their order are: PEG, $MgSO_4.7H_2O$, urea, glycerol, glucose, KH_2PO_4, $CaCl_3.H_2O$, soya bean meal. In define medium the carbon and nitrogen sources plays an important role as a source of precursors for biomass and mineral salts acts as cofactors for the enzymatic reactions in compactin production. The main effect of the components is negative, it indicates that the concentration required for enhancing compactin production is lower than the concentration used in the PB design. Similarly if the effects is positive, the amount of required for the production of compactin was higher than the concentration used in the design.

The pareto plots offers a convenient view of the results obtained by PB design illustrated in Fig 2 the main effects of medium component on the compactin production were presented in Fig 1 This plots is very useful in determining the compactin production at intermediate levels of different combination of independent variable. The pre-optimized was determined based on the main effects. The component having positive main effect were kept the concentration at higher levels and the component which is having negative main effect were kept the concentration at lower level. The variables which are having positive main effects, it means the concentration of glucose, soybean meal, KH_2PO_4, Urea and $CaCl_3.H_2O$ can be increase. The variables which are having negative effects, it means the concentration Glycerol, PEG and $MgSO_4.7H_2O$ can be decrease. The maximum compactin production was 232.11 mg/L was obtained in PB optimization, hence it is proven that PB design is to evaluate the dominant factors present in the medium. Further optimization can be done using Response Surface Methodology (RSM) using above dominant factors evaluated from PB experimental design.

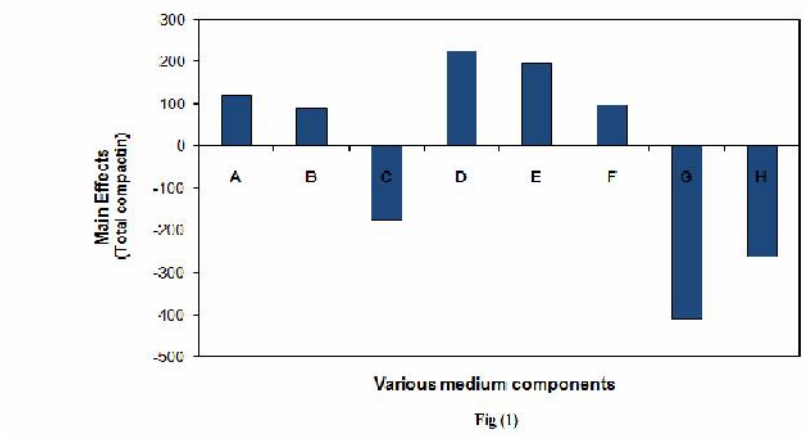

Fig 1. The main effect plot various media components for compactin production through PB design.

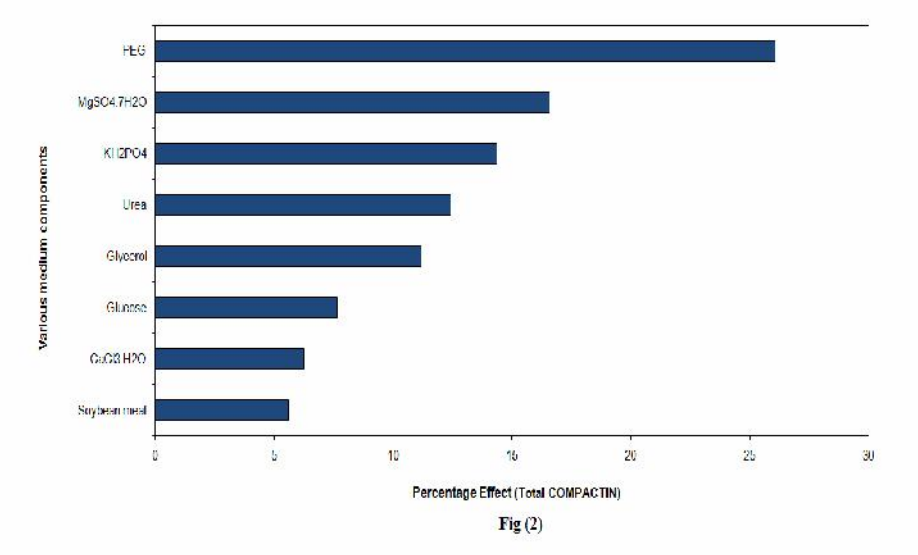

Fig 2. The percentage effect plot for various media components through PB design

Conclusion

Designing the medium was a time-consuming and laborious process involving large number of experiments. The Plackett-Burman experimental design is the preliminary technique for rapid screening of the effects of various medium constituents. Initial various carbon and nitrogen sources have been tested to choose best carbon and nitrogen for the maximum compactin production. PB experimental design was used to evaluate the significance of various medium components and to enhance the compactin production in submerge fermentation. The variables A-H (Table 3) represents the nutrient components, the independent variables and their respective high and low concentration was used in PB optimization study. The maximum compactin production was 232.11 mg/L was obtained in PB optimization study using *P. citrinum*. This technique proved to be valuable in screening a large number of constituents in production media effectively.

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