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Plackett-Burman Statistical Optimization of Media Components for Anti-Mycobacterial Metabolite Production by Marine *Penicillium chrysogenum* DSOA.

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Abstract:

Penicillium chrysogenum DSOA, a strain previously isolated from the sponge *Tedania anhelans* collected off the Indian Ocean had showed potential activity against *Mycobacterium smegmatis.* It is found to be a potential candidate in the search for novel anti-TB drugs, and hence a need to produce active components in larger quantities. One way of accomplishing is this through the optimization of growth conditions including, the incubation period, initial pH, NaCl concentration, and carbon and nitrogen sources. Thus a combination of techniques- classical one factor at a time (OFAT) approach and statistical Plackett Burman approach was used. The OFAT was used to select the ideal carbon and nitrogen sources from a panel, and Plackett Burman used to study the significance of these chosen factors. Glucose and calcium nitrate were found as suitable carbon and nitrogen sources by classical method. Glucose was found to enhance the activity 3.75 fold and calcium nitrate 1.625 fold. Glucose at 1.25% (w/v), calcium nitrate at 1% (w/v), and sodium chloride at 4.25% (w/v) in the medium with incubation period for 21 days at an initial pH of 7 with nutrient broth as basal medium was found to enhance the anti-mycobacterial metabolite production of DSOA. In the case of glycerol asparagine broth as the basal medium, Glucose concentration at 0.5% (m/v), calcium nitrate at 1.5%, sodium chloride at 0.5% or 8% at an initial ph of 11 incubated for 21 days was shown to increase the production of the desired secondary metabolite. The secondary metabolite production was found to be significantly different between the complex media and defined media.

Keywords: Plackett burman, *Penicillium chrysogenum*, marine sponges, carbon and nitrogen sources.

INTRODUCTION

Tuberculosis, a major disease, contributes to a mortality rate 1.5 million annually, according to the WHO report 2014. WHO had already laid out plans to eradicate the disease by 2050 with the development of novel drugs [17]. Until the year 1996, around 80% of the drugs used were either directly sourced from natural products or inspired by them. Abundant bioactive compounds are found in various naturally occurring sources, including plants, microbes and marine organisms [10, 16]. A wide range of microbial diversity exists in the marine ecosystem, either in the planktonic form or as a part of holobionts with sessile marine invertebrates such as coral and sponges [8, 11, 12]. These associated symbionts are known to produce secondary metabolites that are utilized by the host in competition, predation and resistance to pathogens [8, 11, 12, 23]. They can be used as a potential source for novel antibiotics [6]. As much as 10,000 novel marine compounds have been reported during 1990-2009, dominantly sourced from the phyla Porifera and cnidarian [15].

Three isolates from sponges collected in the Florida Keys (USA) produced kocurin, a novel member of thiazolyl peptide class of antibiotics, which was shown to be active against multi drug resistant *Staphylococcus aureus* [21]. Every year around 200 new metabolites are being reported from sponges alone. Few marine compounds have advanced till the clinical trials as well [14]. Halicyclamine A, Lobophorins G, A and B isolated from a marine source also exhibited anti-mycobacterial property [1, 4]. Sterols, terpenes, alkaloids, and peptides are metabolites found to be active against *Mycobacterium tuberculosis* [9, 30].

Optimizaion of the culture conditions is a critical process for enhanced production of antibiotics [7]. The traditional non-statistical approach is highly tedious and leads to misleading interpretations as it is not accurate in accounting for molecular level interactions [17]. Hence experimental designs such as Plackett burman, Response Surface Methodology are used which greatly enhance the yield of product, reduce time, cost and process variability [2].

The Plackett Burman design based on Hadamard matrix can be used to study the effect of a very large number of factors with very less number of observations. This design is also supplemented with centre points in order to test for the non- linearity of the system. Additionally, replicates were also added to account for pure error or random error associated with the experiment [22]. The aim of this study was to optimize significant growth media components in the secondary metabolite production by a marine sponge associated *Penicillium chrysogenum* DSOA. Panels of carbon and nitrogen sources were selected, and the ideal sources were evaluated using the OFAT approach, and Plackett Burman design.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and solvents were purchased from Merck Limited, India; culture media, sugars, organic and inorganic nitrogen sources and growth supplements were purchased from Hi-Media Laboratories Limited, India; and glassware from Borosil Limited, India.

Growth conditions and preliminary screening of crude extract

P. chrysogenum DSOA isolated from *T. anhelans* exhibited anti-mycobacterial activity against *Mycobacterium smegamtis* (Communicated). For preparation of extracts, 1% of seed culture was inoculated in 100 mL of Nutrient Broth (NB) medium and incubated for 15 days under static conditions at 28°C. Bioactive compounds from the culture supernatant were extracted by mixing with equal volumes of chloroform. After intermittent mixing for 2 days, the separated chloroform layer was concentrated using Rota Evaporator (Buchi R250 with V700 vacuum pump, Switzerland), and stored at 4°C. *M. smegmatis* (MTCC6) was used as a test microbe for screening the bioactivity of the extract. *M. smegmatis* was maintained in Luria Bertani Broth (LB) supplemented with glycerol and tween-80 to a final concentration of 4 and 0.1% respectively. The extract (500 µg/disc) was screened for bioactivity against *M. smegmatis* using Standard Kirby- Bauer disk diffusion assay [13] Diameter of Inhibition zones were measured in mm around the disc.

Medium optimization using one-factor-at-a-time (OFAT) classical method

Growth factors such as carbon and nitrogen sources were considered for the enhanced production of secondary metabolites [3, 5]. NB was supplemented with different carbon sources (fructose, glucose, glycerol, raffinose, sucrose; at final concentration 1% w/v) and with different nitrogen sources (inorganic sources such as ammonium acetate, ammonium oxalate, ammonium sulphate, calcium nitrate, imidazole, organic sources such as casein hydrolysate, tryptone, yeast extract; 1% w/v, amino acids such as asparagine, histidine, methionine, phenylalanine, proline; 0.1 mg/mL). The sugars and amino acids were filter sterilized and added to the autoclaved nutrient broth, whereas other nitrogen sources were directly added to the medium before sterilization of the medium [25]. The medium (100 mL) was inoculated with 1% seed culture of the strain DSOA, and incubated for 15 days under static conditions at room temperature. Extracts were collected and disc diffusion assay was performed as described above.

Statistical optimization using Plackett-Burman (PB) design

Initial pH of the medium, incubation period, sodium chloride concentration, carbon and nitrogen sources were considered for the PB design. A 15-run experiment was designed based on factorial design- Plackett Burman, with 12 different combinations and 3 midpoint replicates. To identify the significant influence of the ingredients of media, each variable was set at two levels, high and low level, as shown in Table 1, with two basal media- NB and the glycerol-asparagine media (glycerol- 1% (v/v), L-asparagine (1g/L), di-potassium phosphate (1 g/L) and trace salt solution (1 ml/L) containing ferrous sulphate heptahydrate (0.001 g/L), manganese chloride tetrahydrate (0.001 g/L) and zinc sulphate heptahydrate (0.001 g/L) adjusted to the pH of 7.4 at 25°C). The basal media were supplemented with various factors and subjected to incubation as per Table 2. The secondary metabolites were extracted from culture supernatants of every experimental run with chloroform. The bioactivity of the extracts were assayed against *M. smegmatis* in MTT assay.

MTT assay

The anti-mycobacterial activity of the extracts of every experimental run was determined by MTT assay [18, 27]. Briefly, LB (100 µL) was dispensed into each well of 96-well titer plate followed by addition of 500 µg of extracts from experimental run to respective wells. 100 µL of *M. smegmatis* containing approximately 5 x 10^4 CFU was dispensed into the wells and incubated for 48 hours at 37°C [27]. LB and *M. smegmatis* culture without extract were used as controls. Standard MTT assay was performed as described [18]. Ten micro liters of MTT solution (5 mg/mL working solution was prepared in 1X PBS of pH 7.2) was added to each well after the incubation period and kept overnight at 37°C for incubation. Added 50 µL of formazan dissolution buffer (1:1, 50% dimethyl formamide and 20% SDS) to each well and incubated further for 3 hrs. The results from the MTT assay were inferred by recording the absorbance at 490 nm in multimode reader (Infinite-M200, TECAN, Switzerland). The results were used for testing the significance of parameters according to the PB design of experiments.

RESULTS & DISCUSSIONS

Optimization of the carbon source and nitrogen source

Various carbon and nitrogen sources were evaluated for the enhanced production of anti-mycobacterial metabolite by DSOA. The carbon and nitrogen sources were selected based on their effect on production of bioactive compounds as reported [19, 20, 24, 25, 29]. Glucose supplementation enhanced the anti-mycobacterial production by 3.75 fold, followed by glycerol and sucrose by 2.5 fold (Fig.1 & 2). Glucose, sucrose and glycerol have been previously used to enhance the antibiotic production. As much as 30 secondary metabolites have been shown to get repressed by the interference with carbon sources, thus the carbon sources were optimized [20]. Glucose repress the formation of aminoglycoside antibiotics such as streptomycin, kanamycin, neomycin, istamycin and gentamycin. [20, 25, 29]. Among the various nitrogen sources, calcium nitrate enhanced the production by 1.625 fold (Fig. 1 & 2), followed by casein hydrolysate, 1.25 fold. The supplementation of calcium nitrate (nitrogen source) to the culture medium enhanced the production of secondary metabolites. Supplementation with amino acids suppressed the production of anti-mycobacterial metabolites. The extracts did not exhibit any activity against the test organism. Nutritional conditions were optimized for the antifungal metabolite production by *S. lavendulae* and *S. fulvissimus* and reported the maximum metabolite activity when the strains were provided with calcium nitrate as a nitrogen source [26]. The nitrogen sources are crucial in the production of enzymes associated with the synthesis of primary and secondary metabolites. Studies have shown the repression of antibiotic production by nitrogen sources, especially ammonium; hence it is desirable to optimize the ideal nitrogen source [28, 31]. Glucose and calcium nitrate tetrahydrate were carried further for the design of experiments using 15-run PB.

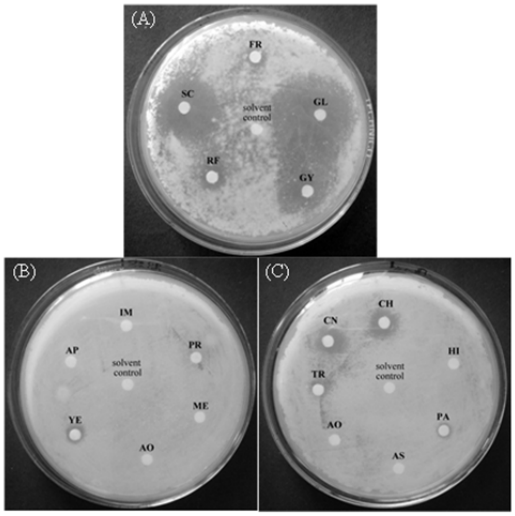

Fig 1: Antibacterial assays of chloroform extracts of strain DSOA grown at various carbon and nitrogen sources. Inhibition zones were observed to the extracts against M. smegmatis. Physiological parameters studied for the experiment are A) Carbon sources, B and C) Nitrogen sources. Different carbon sources include fructose (FR), glucose (GL), glycerol (GY), raffinose (RF), sucrose (SU); nitrogen sources include ammonium acetate (AA), ammonium oxalate (AO), ammonium sulfate (AS), calcium nitrate (CN), imidazole (IM), casein hydrolysate (CH), tryptone (TR), yeast extract (YE), asparagine (AP), histidine (HI), methionine (ME), phenyl alanine (PA), proline (PR).

Table 1. High and low values of the five parameters.

Variable	Parameter	+(High level)	-(Low level)	Middle point (M)
A	Incubation period (Days)	21	9	15
B	Initial pH	11	3	7
C	Glucose (m/v %)	2	0.5	1.25
D	Calcium Nitrate (m/v %)	1.5	0.5	1
E	NaCl (m/v %)	8	0.5	4.25

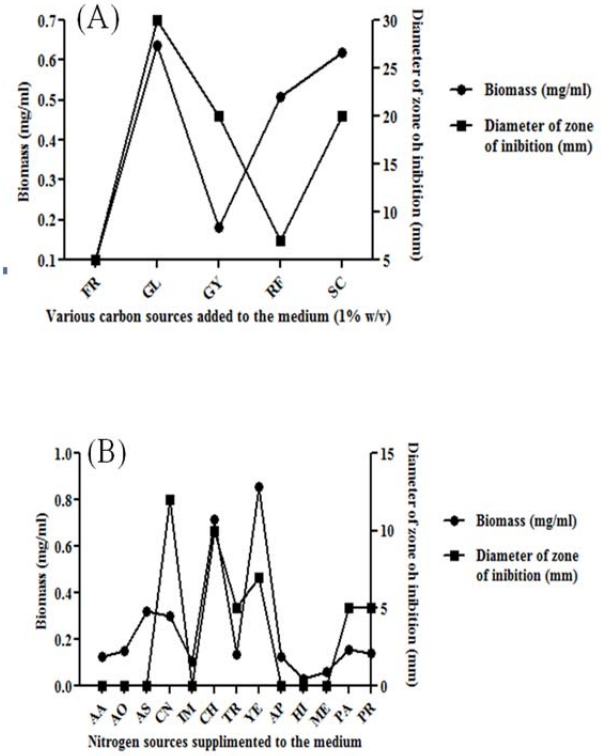

Fig 2: Effect of carbon and nitrogen sources on the growth and secondary metabolite production of strain DSOA. Biomass (in mg/ml) and diameter of inhibition zone (in mm) factors were represented against each parameter. Parameters studied for the experiment are A) Carbon sources and B) Nitrogen sources. Different carbon sources include fructose (FR), glucose (GL), glycerol (GY), raffinose (RF), sucrose (SU); nitrogen sources include ammonium acetate (AA), ammonium oxalate (AO), ammonium sulfate (AS), calcium nitrate (CN), imidazole (IM), casein hydrolysate (CH), tryptone (TR), yeast extract (YE), asparagine (AP), histidine (HI), methionine (ME), phenyl alanine (PA), proline (PR).

Table 2. Experimental Design for media optimization. % Death calculated based on MTT assay. NB: Nutrient broth as basal media, GA: Glycerol asparagines broth as basal media. A- Incubation period (days), B- pH, C- Glucose (m/v %), D- Calcium Nitrate (m/v %), and E- Sodium Chloride (m/v %). M: Centre point.

Run	A	B	C	D	E	% Death for NB	% Death for GA
1	-	+	+	+	-	77.94	68.61
2	+	+	+	-	+	92.95	74.26
3	-	-	-	+	+	58.77	27.1
4	+	+	-	+	+	96.87	91.38
5	-	+	-	-	-	80.89	21.52
6	M	M	M	M	M	99.43	51.08
7	+	-	+	+	-	79.32	51.76
8	-	-	+	+	+	70.33	68.92
9	+	-	+	-	-	80.41	68.32
10	-	+	+	-	+	76.23	71.6
11	+	-	-	-	+	94.66	58.6
12	M	M	M	M	M	99.397	57.1
13	-	-	-	-	-	67.78	88.21
14	M	M	M	M	M	99.392	58.45
15	+	+	-	+	-	94.34	90.08

Table 3. Estimated effects and coefficients of the parameters for anti-mycobacterial metabolite production by *Penicillium chrysogenum* DSOA using Plackett Burman design of experiment with nutrient broth as basal medium.

Variable	Term	effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant		35.24	0.958	56.48	0.000	
A	Incubation period	11.885	0.990	0.958	6.21	0.000	Significant
B	Initial pH	7.572	0.946	0.958	3.95	0.004	Significant
C	Glucose	-1.798	-1.2	0.958	-0.94	0.375	*
D	Calcium Nitrate	-1.708	-1.71	0.958	-0.89	0.398	*
E	NaCl	1.018	0.136	0.958	0.53	0.609	*
M	Centre point		12.40	2.14	5.79	0.000	

. *: Insignificant.

Table 4. Estimated effects and coefficients of the interaction between parameters for anti-mycobacterial metabolite production by *Penicillium chrysogenum* DSOA using Plackett Burman design of experiment with nutrient broth as basal medium.

Variable	Term	Effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant		31.7371	0.006	13568.31	0.000	
A	Incubation period	18.8866	2.67734	0.00843	1120.28		Significant
B	Initial pH	5.7033	2.40087	0.00843	338.30	0.000	Significant
C	Glucose	-2.58167	20.5975	0.00843	-153.13	0.000	Significant
D	Calcium Nitrate	-5.29833	9.0717	0.00843	-314.28	0.000	Significant
E	NaCl	-4.3433	-2.87022	0.0133	-162.94	0.000	Significant
AB	Incubation period * initial pH	0.9516	0.019826	0.00843	56.45	0.000	Significant
AC	Incubation period * Glucose	-11.64	-1.29333	0.0126	-460.29	0.000	Significant
AD	Incubation period * Calcium Nitrate	-1.6483	-0.27472	0.00843	-97.77	0.000	Significant
AE	Incubation period * NaCl	6.8733	0.152741	0.00843	407.70	0.000	Significant
BC	Initial pH * Glucose	-2.50167	-0.41694	0.00843	-148.39	0.000	Significant
BD	Initial pH * Calcium Nitrate	-5.8567	-1.46417	0.0133	-219.71	0.000	Significant
M	Centre point		18.5322	0.0133	1390.46	0.000	Significant

The interactions: Initial pH*NaCl, Glucose*Calcium Nitrate, Glucose*NaCl, and Calcium Nitrate*NaCl could not be computed and hence were not tabulated.

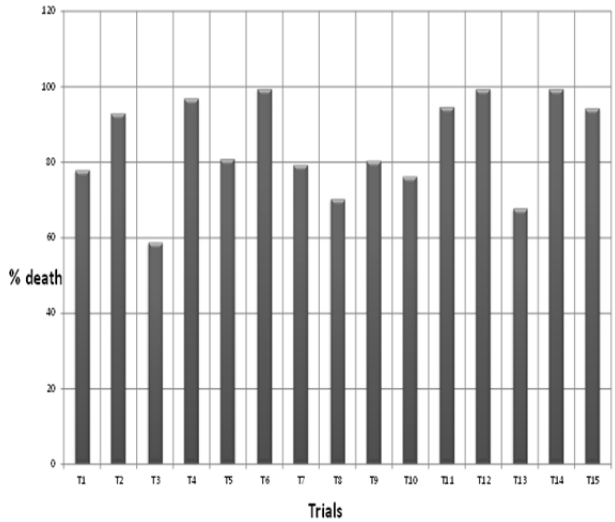

Fig 3: Inhibition with nutrient broth as basal medium based on MTT assay.

Medium optimization using Plackett-Burman experimental design

Improving media composition using statistical methods have proved to be a useful tool for the consideration of several factors between two levels, for which Plackett Burman experimental setup was designed with 5 parameters for optimization. The activity of concentrated secondary metabolites was determined by MTT assay. The percentage death of the cells after the addition of extracts is tabulated in Table 2. The antagonistic potential was observed maximum in run order 6, 12 and 14, followed by 2, 4, 11 and 15 runs with NB as basal medium, and 4 and 15 runs with GA as basal medium, had showed an optimized activity as shown in Fig. 3 & 4.

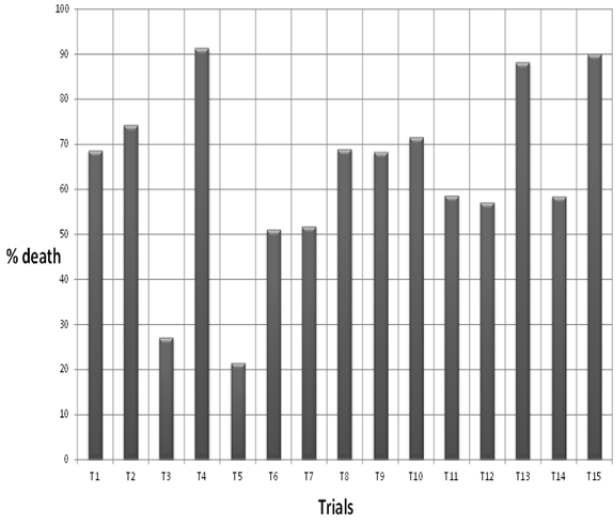

Fig 4 : Inhibition with glycerol asparagines broth as basal medium based on MTT assay.

Table 5. Estimated effects and coefficients of the parameters for anti-mycobacterial metabolite production by *Penicillium chrysogenum* DSOA using Plackett Burman design of experiment with glycerol asparagine broth as basal medium.

Variable	Term	Effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant		21.5	4.63	9.4	0.000	
A	Incubation period	9.86	0.821	4.63	1.07	0.318	*
B	Initial pH	6.08	0.76	4.63	0.66	0.53	*
C	Glucose	2.96	1.98	4.63	0.32	0.757	*
D	Calcium Nitrate	1.71	1.71	4.63	0.19	0.858	*
E	NaCl	0.37	0.05	4.63	0.04	0.969	*
M	Centre point		-4.8	10.3	-0.46	0.658	

*: Insignificant.

Table 6. Estimated effects and coefficients of the interaction between parameters for anti-mycobacterial metabolite production by *Penicillium chrysogenum* DSOA using Plackett Burman design of experiment with glycerol asparagine broth as basal medium.

Variable	Term	Effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant		189.5	1.13	57.41	0.000	
A	Incubation period	7.29	-5.44	1.6	2.28	0.151	*
B	Initial pH	-3.69	-17.92	1.6	-1.15	0.369	*
C	Glucose	31.41	40.78	1.6	9.8	0.010	Significant
D	Calcium Nitrate	-10.63	-112	1.6	-3.32	0.080	*
E	NaCl	-0.21	-0.53	2.53	-0.04	0.971	*
AB	Incubation period * initial pH	30.67	0.6391	1.6	9.57	0.011	Significant
AC	Incubation period * Glucose	-15.40	-1.711	2.4	-3.20	0.085	*
AD	Incubation period * Calcium Nitrate	21.41	3.569	1.6	6.68	0.022	Significant
AE	Incubation period * NaCl	1.51	0.0336	1.6	0.47	0.684	*
BC	Initial pH * Glucose	1.51	0.832	1.6	1.56	0.26	*
BD	Initial pH * Calcium Nitrate	4.99	6.84	2.53	5.40	0.033	Significant
M	Centre point	27.34	-9.49	2.53	-3.75	0.064	

*: Insignificant. The interactions: Initial pH*NaCl, Glucose*Calcium Nitrate, Glucose*NaCl, and Calcium Nitrate*NaCl could not be computed and hence were not tabulated.

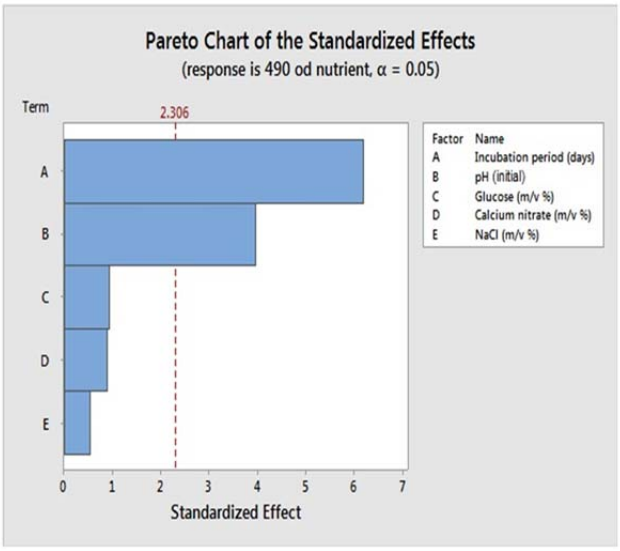

Fig 5 : Pareto chart for individual factors with nutrient broth as basal medium.

Statistical analysis using Minitab 17

All the experiments were carried out in triplicates and the data represented are a mean of independent measurements. Based on a Pareto chart, the significance of the parameters and interactions were distinguished. The absolute value for the effects has been depicted on the Pareto chart and a standardized reference line also included based on a 95% confidence level. The effects whose value had crossed the reference line were considered statistically significant ($p<0.05$). Additionally, the influence of the parameters, either synergistic or antagonistic was understood with the help of the coefficients/effect. A positive regression coefficient in the tabular columns indicated a synergistic effect on the secondary metabolite production whereas a negative coefficient indicated an antagonistic effect. The effect of the parameters on the growth of the DSOA was analyzed considering the percentage cell death as the response. The obtained results were subjected to regression analysis and the analysis of variance (ANOVA) were performed on the data using the Minitab 17 software. The analysis was done in two ways; Case 1: considering only the individual parameters, and Case 2: considering two way interactions between the factors.

When NB was the basal medium (Fig. 5), only the incubation period and initial pH of the medium had a significant influence on the desired response. The results from Table 3 indicated that the high level of incubation period, initial pH and NaCl concentration and the low level of glucose and calcium nitrate concentration were desirable for the production of the secondary metabolite. When the two way interactions between the factors was considered (Fig. 6 and Table 4), all the calculated interactions and the individual parameters were found to be significant. High levels of incubation period and initial pH and low levels of Glucose, calcium nitrate and NaCl were desirable for the production of the anti-mycobacterial secondary metabolite.

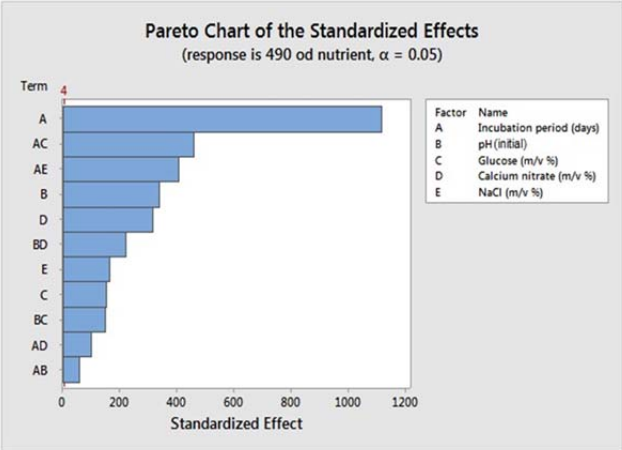

Fig 6 : Pareto chart for factors along with two way interactions with nutrient broth as basal medium.

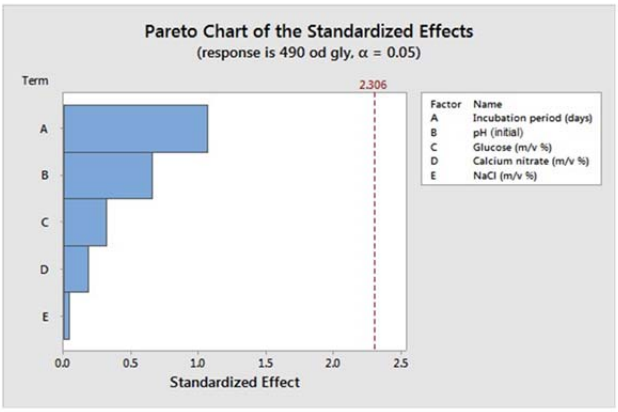

Fig 7: Pareto chart for the individual factors with glycerol asparagine broth as basal medium.

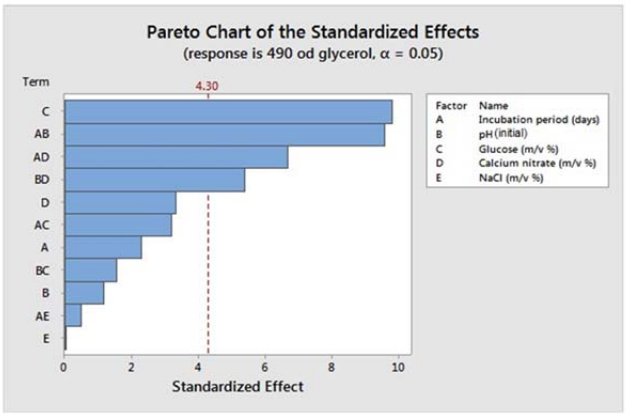

Fig 8 :Pareto chart for two way interaction between the factors with glycerol asparagine broth as basal medium.

In case of glycerol asparagine broth as basal medium (Fig. 7 and Table 5), none of the parameters were found to be significant. However as per the effects, the high level of all the five parameters was required for the desirable response. When the two way interactions between the factors were considered (Fig. 8 and Table 6), glucose, Incubation*pH, Incubation*Calcium nitrate and Initial pH*calcium nitrate were significant.

Thus from the above experimentation, the differences in the responses between a complex media and a defined media can be very well understood. Even in the absence of additional growth supplements, a complex media is able to support the metabolic processes in an organism, whereas, one needs to optimize proper growth factors to achieve the desired response when using a defined media. In the case of nutrient broth as basal media, 7 out of 15 trials showed more than 90% activity, whereas in the case of glycerol asparagine broth as basal media, only 2 out of 15 trials showed 90% activity.

Culture conditions were optimized successfully for metabolite production using the statistical methods. The effective combinations of glucose, calcium nitrate and sodium chloride in the medium with optimized incubation period and initial pH had influenced the potential metabolites production. These findings will be useful in the medium formulation for producing new therapeutic compounds in large-scale for exploiting their biotechnological potential. Moreover, a comparative study between a complex medium and a defined medium had emphasized the need for optimizing culture conditions in order to achieve the desired result.

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