Taguchi's experimental design for optimizing the production of novel thermostable polypeptide antibiotic from *Geobacillus pallidus* SAT4

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Abstract: Polypeptide antimicrobials used against topical infections are reported to obtain from mesophilic bacterial species. A thermophilic *Geobacillus pallidus* SAT4 was isolated from hot climate of Sindh Dessert, Pakistan and found it active against *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* NCTC 10400 and *Pseudomonas aeruginosa* ATCC 49189. The current experiment was designed to optimize the production of novel thermostable polypeptide by applying the Taguchi statistical approach at various conditions including the time of incubation, temperature, pH, aeration rate, nitrogen, and carbon concentrations. There were two most important factors that affect the production of antibiotic including time of incubation and nitrogen concentration and two interactions including the time of incubation/pH and time of incubation/nitrogen concentration. Activity was evaluated by well diffusion assay. The antimicrobial produced was stable and active even at 55°C. Ammonium sulphate (AS) was used for antibiotic recovery and it was desalted by dialysis techniques. The resulted protein was evaluated through SDS-PAGE. It was concluded that novel thermostable protein produced by *Geobacillus pallidus* SAT4 is stable at higher temperature and its production level can be improved statistically at optimum values of pH, time of incubation and nitrogen concentration the most important factors for antibiotic production.

Keywords: *Geobacillus pallidus*, antibiotic, Thermostability, Taguchi approach.

INTRODUCTION

The antibacterial compounds broadly dispersed in nature and are involved to play a significant part in controlling and balancing the ecology of the soil/lands, water reservoir, sewage systems, and dung microbial communities. A number of greatly used natural compounds have been synthesized from a trivial group of microbes including the genera of Penicillium, Streptomyces, Cephalosporium, Micomonospora and Bacillus species (Zinsser *et al*., 1988). Polypeptide antibiotics, usually synthesized at the early stages of sporulation, are produced by many strains of the genus Bacillus. These species produces the most important polypeptide antibiotics are: bacitracin, polymyxin, gramicidin, subtilin, bacillin, pumilin, mycobacillin, and colistin. Berdy in 1974 reported that these polypeptide antibiotics produced by Bacillus species was 167 and out of this total, 66 diverse peptide antibacterial molecules were synthesized by strains of *Bacillus subtilis* and 23 were bio active compounds of *Bacillus brevis*. Polypeptide antibiotic compounds including polymyxin and the closely related other peptide compounds like colistin, bacitracin, the tyrothricin complex and gramicidin S have been used, to some extent, for antimicrobial cure. These antibiotics synthesized by Bacilli are effective against Gram positive bacterial species; however polymyxin-B colistin and circulin showed activity against Gram-negative bacteria, while bacillomycin, mycobacillin, and fungistatin compounds are active against yeasts and molds (Anderson *et al*., 1972). Low molecular weight to high molecular polypeptide antibiotics, synthesized via the non-ribosomal biosynthetic pathway using particular enzymes called peptide synthetases, have been reported from a large number of Bacillus species. These bio active polypeptide compounds have a diverse array of significant biological activities effects like antibacterial, antifungal, antiviral and anti-tumoral activities (Cane *et al*., 1998). It is a recognized that protein antibiotics and extra-cellular enzymes synthesis by microbes is greatly affected by nutritional ingredient particularly carbon and nitrogen components (Hanlon *et al*., 1982; Kaur *et al*., 2001; Kole *et al*., 1988a; Kole *et al*., 1988b), minerals (Varela *et al*., 1996) and physical-aspects such as pH assessment, temperature range, inoculum mass (Nehete *et al*., 1985) presence of oxygen (Moon and Parulekar, 1993) and time of culture incubation (Nehete *et al*., 1985; Oberoi *et al*., 2001). The effect of growth factors including carbon, nitrogen, and vitamin concentrations, presence of minerals, physical, chemical and other fermentation factors have been assessed and reported in various studies which has discussed the optimum conditions of these factors required for optimization of enzymes and polypeptide antibiotics synthesis (Varela *et al*., 1996; Adinarayana, Ellaiah, 2000 and Awais, 2007). Geobacillus species (Shida *et al*., 1996), an aerobic and spore forming unicellular microbe, categorized by its capability to yield small narrow and lamella shaped para-

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sporal attachment next to the spore and has been recognized to comprise microbial strains lethal to some invertebrates (Favret and Yousten, 1985; Rivers *et al*., 1991). Similarly, few strains of this specie has been found to synthesize the therapeutically important compounds such as spergualin (Nemoto *et al*., 1987; Umezawa and Takeuchi, 1987), and bacithrocins A, B and C (Kamiyama *et al*., 1994), and also including a peptide antibiotic with cyanolytic activity (Krachkovskii *et al*., 2002). This strain SA14 of this specie has been reported to produce 116kDa antibiotic protein, effective against *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans (*Choopan *et al*., 2008).

Thermostability is the feature of chemical molecules to cope the irreversible modification in its chemical or physical properties even at higher temperatures. Majority of life forms on this earth exist at temperatures of less than 50°C, normally between 15°C to 50°C. At higher temperatures or above this value, high temperature energy may be the source of un-folding of the protein forms and even such change may be irreversible, a situation reasonably harmful to continuing the biological functions. There are some thermophilic species that live even at higher temperatures. Investigating the homology and similarity in these protein structures present in the thermophiles and other related species showed only minor differences and one important change is the occurrence of extra hydrogen bonds interaction in the thermophilic proteins which means that these protein forms are more resistant to unfolding.

Statistical techniques have benefits over orthodox practices in calculating the precise outcomes mainly due to application of important codes of statistics assessment, random data streaming, and duplication. One of the important and widely used scale-up processes is the response-surface and optimization, mainly established based on full factorial central composite design (Box *et al*., 1975). Many fermentation procedures have been improved using this approach (Sen and Swaminathan, 1997; Smith *et al*., 1997; Teruel *et al*., 1997). However, this arithmetical investigational study is only associated with the number of variance factors but is not linked to the statistical factorials (Box *et al*., 1975). Therefore it has not yet been commonly used in the biological fields. The newly established Taguchi technique (Joseph and Piganatiells, 1988) built on orthogonal-arrays systems (OAs) offers 3-stages of off-line quality control features like system plan, parameter evaluation and tolerance design phase. System strategy evaluation supports to find the investigational levels of design features while parameter design shows the factor level, provides the paramount effects of the process, whereas tolerance design phase advantages in improving the tolerance of the elements that considerably effect the product development. The Taguchi practices not only supports in significant saving in time and cost but also takes us to a more fully advanced industrialized procedures.

The current work aimed to produce and characterize the thermostable polypeptide antibacterial compound from *Geobacillus pallidus* strain SAT4 that was effective against Gram-positive bacteria. The identification of this strain is resulted by using the morphological, biochemical and molecular techniques. The production was optimized at different conditions by Taguchi method to comprehend the accumulative effects of the variance factors including time of incubation, temperature, pH, aeration rate, nitrogen, and carbon concentrations (fig. 1).

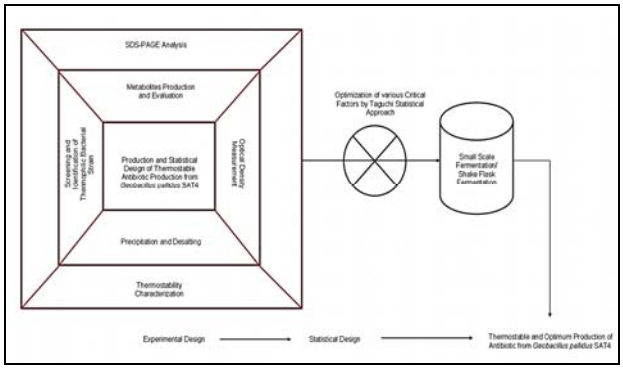

Fig 1: Schematic design of the experiment for production of thermostable antibiotic.

MATERIALS AND METHODS

Screening and identification of Bacterial strain

Thermophilic bacterial strain used in this study was collected from Thar desserts, Sindh, Pakistan, isolated and purified through streak plate and pour plate techniques using nutrient agar media. Isolated colony was identified on the basis of standard morphological, biochemical tests (Bergey and Holt, 1994) and 16S rRNA gene sequencing technique. For biochemical tests, the isolate was evaluated by using the API 50CHB V4.0 kit (BioMerieux France; Lot No. 833022401). The results after 24 hours were transferred into a bio- Me´rieux identification software database (Apiweb^{TM}; BioMerieux SA).

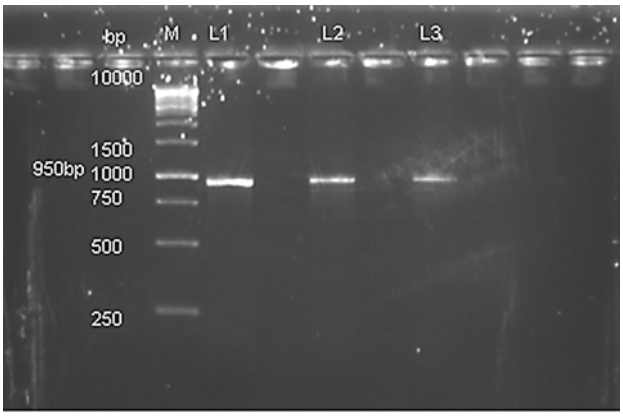

Fig 2: Size of Amplicon (PCR product).

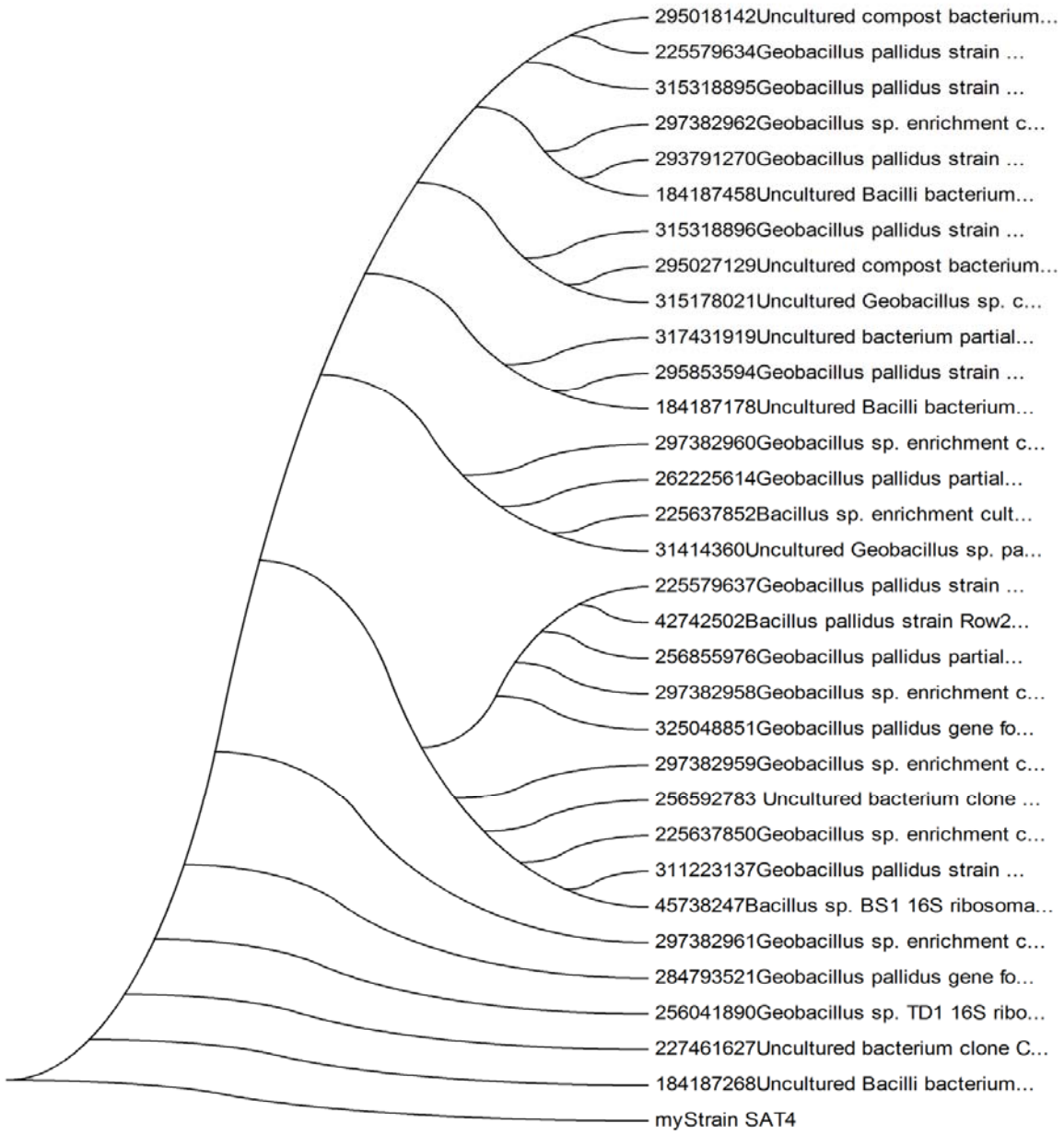

Fig 3: Evolutionary relationships of 32 taxa (linearized)
The evolutionary history was concluded using the NJ method. The related tree with the sum of branch length = 0.01673580 is shown here. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 419 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.0.

Molecular Characterization and Phylogenetic study

A molecular genetic approach was used to identify the bacterial isolate. Molecular DNA (deoxyribonucleic acid) was extracted from bacterial cultures using Wizard genomic Kit (Promega, Madison, USA) according to the manufactures' specifications. Polymerase chain reaction amplification and sequencing of bacterial gene was carried out using a Takara 16S rDNA bacterial Identification kit. 1 µl of the extracted DNA was amplified with universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R907 (5'-CCGTCAATTCCTTTRAGTTT-3'), generating a PCR product having 950bp size. Extracted DNA was visualized by gel electrophoresis on 0.8 % agarose gel and 5 µl of

PCR products on 1.5 % agarose gel stained with ethidium bromide (0.5 mg/ml) in 1x TAE buffer. 1kbp DNA ladder (Fermentas GeneRuler^{TM}, #SM0313) was used for PCR products (fig. 2). The gel was then observed for bands under UV using gel-dock imaging system (BioRad, Milan Italy). Sequencing of PCR products was performed and analyzed in both directions using an ABI Prism 310 automated DNA sequencer using BigDye Terminator cycle sequencing kit (PE Applied and Biosystem USA). That kit contained a BigDye Terminator tube, filled with 10 µl of pinkish solution containing 2 µl of primer and 8 µl of BigDye Terminator Reagent. 10 µl of purified PCR product was transferred to Big Dye Terminator tube. Then samples were sequenced according to protocol similar to PCR protocol. Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) was used to align the bacterial 16S rDNA sequence with hundreds of known different available sequences in the database, and percent homology score was generated to identify bacteria. Phylogenetic tree was constructed by the neighbor joining technique using the software Mega 4.0.2 package.

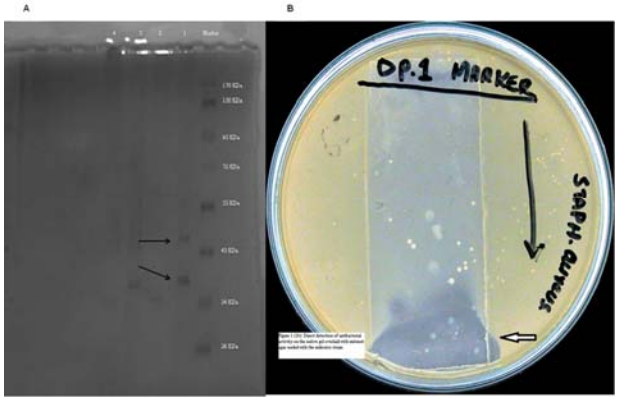

Fig 4: SDS-PAGE analysis
(A) Purification through SDS-PAGE; arrow indicates the fractionated proteins in the sample compared with the Marker (in Kilo Dalton). (B) Direct detection of antibacterial activity on the native gel overlaid with nutrient agar plate seeded with the *Staphylococcus aureus* as indicator strain.

Antibacterial metabolites production

The metabolites produced from thermophilic Geobacillus specie were evaluated against *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* NCTC 10400 and *Pseudomonas aeruginosa* ATCC 49189. 100 ml of *growth* media (composition in g/l: 1.0g lab-lemco powder, 2.0g yeast extract, 5.0g peptone, 5.0g sodium chloride, 15g agar) was prepared in 250 ml flask, inoculated with a fresh culture of Bacillus specie SAT4 and incubated at 55°C for 24 hours in an orbital shaker at 180rpm. 100 ml of production media (composition in g/l: 5.0g L-glutamic acid, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.2g MgSO4.7H2O, 0.01g $MnSO_4$.H2O, 0.01g NaCl, 0.01g $FeSO_4.7H_2O$, 0.01g $CuSO_4.7H_2O$, 0.015g $CaCl_2.2H_2O$, 2 % glucose) was inoculated with (Bushra *et al.*, 2007) 10 % (V/V) inoculum. For antibacterial metabolite production, flask was incubated at 55°C for 24 hours in orbital shaker at 180rpm and activity was checked through well diffusion assay (Gatsing *et al*., 2006). Growth was measured by taking optical density at wavelength of 650nm by using the UV-Spectrophotometer 8453 (Agilent USA) and antibacterial metabolites production ability was evaluated at various optimizing parameters through well diffusion assay (table 4, fig. 5).

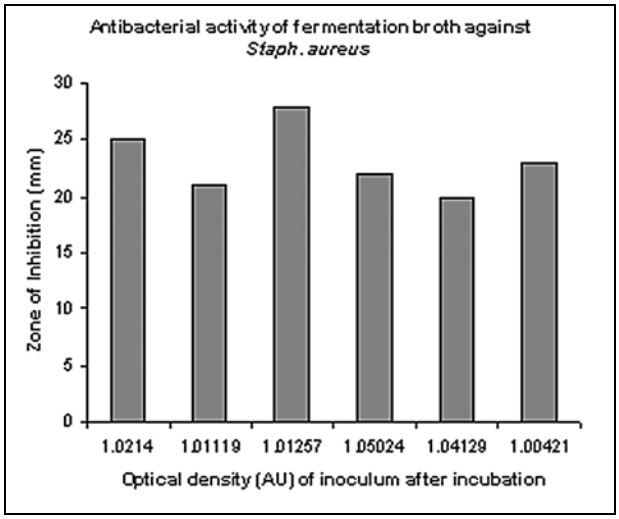

Fig 5: Optical density of inoculum measured after incubation showed greater zone of inhibition at given conditions against *Staphylococcus aureus* showing increased production of polypeptide antibiotics.

Optimization of parameters

The antibacterial metabolite production was optimized at different parameters. These parameters included the time of incubation, pH, temperature, aeration rate, nitrogen, and carbon concentrations. Incubation time was evaluated from 24 to 144 hours; pH was studied by taking the values from 4 (acidic) to 9 (basic); temperature ranged from 45 to 60°C; the glutamic acid used as a source of nitrogen varied from 0.25 to 2%; and carbon concentration by taking the glucose from 0.25 to 3%. The optimizing levels were confirmed through well diffusion assay (Awais *et al.*, 2008) and biological activity was observed by calculating the zone of inhibition.

Precipitation and Desalting

Precipitation of protein antibiotic was carried out by ammonium sulphate at the concentration of 50 %. After sufficient shaking, solution was placed in the cold for one hour and then precipitates were collected by centrifugation at 14000 rpm for 15 minutes at 4°C. The precipitates, which contains the antibacterial substance was then resuspended in 0.05M phosphate buffer of pH 6. The 0.05M phosphate buffer of pH 6 was used to dialyze the precipitates in dialysis tubing of 10KDa cut off.

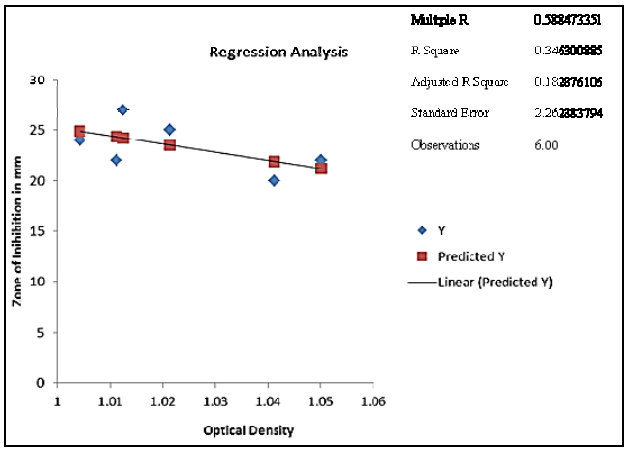

Fig 6: Regression analysis scatter graph between dependent and independent variables (optical density and zone of inhibition in mm).

SDS-PAGE Analysis

Most of the analyzed antibacterial agents are found to be proteins or conjugates of proteins. With the aim of monitoring purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12 % of resolution gel and 5 % of stacking gel (Lee *et al*., 1987). Sample was prepared by dissolving it with equal proportion of sample buffer and loaded onto several wells along with molecular weight standards (Fermentas, PageRuler, Prestained Protein Ladder, # SM 0671). One half of the PAGE gel was stained and marked with coomassie brilliant blue dye according to the method of Lee *et al*. (1987) while the other half was evaluated directly for antibacterial activity by the modified-test described by Bhunia *et al*. (1992).

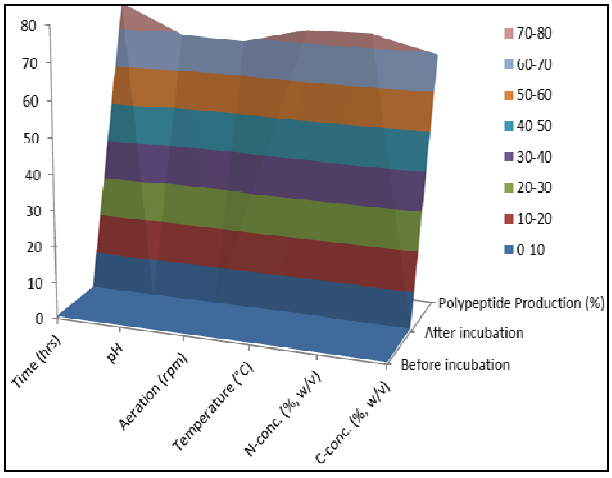

Fig 7: Three-dimensional response surface and the responding contour plot for the optimum production of polypeptide antibiotics at time of incubation (hrs), pH, and nitrogen concentrations [N: nitrogen; C: carbon; Optical density before and after incubation; Time: time of incubation].

Taguchi analysis for experimental study

In this experiment, all six columns were designed with various vital factors and each factor was assigned with five levels (table 3). Table 5 shows the layout of the L25 (5^6) OA used in the present study. The procedure of the OA experimental studies resolves the parameters affect relatively straightforward (Taguchi *et al*., 2004). The figurative description of these arrays specifies the core evidence on the size of the trials, e.g. L25 has 25 trials. Taguchi approach was used to recognize the influence of each factor for maximum antibacterial metabolite production and determination of the optimum conditions, Qualitek-4 software pack Nutek, Inc., USA was also used for this purpose. The potential combinations of all trials were conducted and polypeptide antibacterial metabolites production was proceeded through shake flask fermentation (table 6). In order to remove the effect of unknown nuisance factors on the antibiotic production during our study, random sampling was used and experimental steps were performed in triplicates.

Thermostable characteristics

Thermostability of antibacterial metabolites was characterized by keeping the samples at various temperatures (45, 50, 55, and 60°C) for one hour, and activity was measured through the well diffusion assay against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus and Pseudomonas aeruginosa*. 55°C was found to be stable for best activity of these metabolites (table 3).

RESULTS

Taxonomic Characterization of Bacterial Isolate

Morphologically, isolate was a Gram-positive rod shaped, motile and spore forming while biochemical studies indicated that citrate utilization, oxidase, starch and casein hydrolysis were positive while indole, catalase and gas production tests were negative with the positive fermentation of glucose showing acid production. API 50CHB combined with API 20E kit showed 98.4 % significant taxon was identified as *Geobacillus pallidus strain* SAT4. *Geobacillus pallidus* fermented the carbohydrate molecules that includes: Glycerol, D-glucose, D-fructose, D-mannose; D-mannitol; D-Sorbitol; Methyl alphaD-glucopyranoside *N*-acetylglucosamine; amygdalin; arbutin; esculin ferric citrate; salicin; D-cellobiose; Sucrose; D-Trehalose; L-Fucose tests (bioMe´rieux, Inc.). The test profile of this *specie* is summarized in table 1.

Molecular Characterization

According to blast outcome at NCBI server, this thermophilic bacterium is a member of the *Firmicutes* that showed 98 % homology with *Geobacillus pallidus* (Accession number AB548614.1) at nucleotide positions from 443 to 874. Evolutionary tree was generated by the

well-known Neighbor Joining (NJ) method and phylogenetic relationship was analyzed (fig. 3).

Table 1: Identification by Morphological, Biochemical and API 50CHB Kit with percentage of positive tests after 24 hours

Test		Result	
Grams Staining		+	
Shape		Rods	
Spore formation		+	
Motility		+	
Indole Production		_	
Citrate Utilization		+	
Oxidase Test		+	
Catalase Test		_	
Starch Hydrolysis		+	
Casein Hydrolysis		+	
Gas Production from Glucose		Gas production –ive, Acid production +ive	
API 50CHB Tests that were positive for [a]			
GLY +	GLU +	FRU +	MNE +
MAN +	SOR +	MDG +	NAG +
AMY +	ARB +	ESC +	SAL +
CEL +	SAC +	TRE +	LFUC +
Significant Taxon 98.4 % *Geobacillus pallidus* SAT4			

Indications: + sign indicates the positive reaction (color change), [a] Only the tests with a positive result are included here (remaining tests were negative). GLY, ; GLU, D-glucose; FRU, D-fructose; MNE, D-mannose; MAN, D-mannitol; SOR, ; MDG, ; NAG, *N*-acetylglucosamine; AMY, amygdalin; ARB, arbutin; ESC, esculin ferric citrate; SAL, salicin; CEL, D-cellobiose; SAC, ; TRE, ; LFUC

Purification of antibacterial metabolites

The polypeptide antibiotic was recovered by using the ammonium sulphate precipitation technique and it was desalted. Precipitation of protein was at 50 % ammonium sulphate concentration. The activity of this novel protein at various temperatures is shown in table 2. The refinement procedure was trailed by SDS-PAGE run. Bands were appeared (fig. 4A) against Pre-stained Protein Ladder. The antibacterial activities of these bands were checked by the modified test. SDS-PAGE showed that the band at the position of 37kDa made a clear and distinct zone of inhibition (fig. 4B) when gel overlaid with *Micrococcus luteus* ATCC 10240, lawn and incubated at 37°C for 24 hours.

Table 2: Thermostability of antibacterial metabolite against indicator strains at different temperatures (diameter in mm)*

Indicator strains	Origin	45 °C	50 °C	55 °C	60 °C
Staphylococcus aureus	ATCC 6538	17	20	22	18
Micrococcus luteus	ATCC 10240	18	22	25	19
Bacillus subtilis	NCTC 10400	14	16	18	16
Pseudomonas aeruginosa	ATCC 49189	6	8	10	8

* The diameter of stainless steel borer was 6mm

Analysis of optimizing parameters using Taguchi method

The Taguchi method includes to reduce the variation in a process through robust design of experiments whose performance is least affected by these noises. The parameters which were optimized include time of incubation at 48 hours, pH of the medium at 5, temperature at 55°C, aeration rate at 180 rpm, nitrogen concentration at 1.5 % and carbon concentration at 2.0 %. Analysis of results leads to a response model in which the relationship of each variable (i.e., factor) towards the response, as well as the interactions between factors, is shown. In regression with a single independent variable, the coefficient exhibits how much the dependent variable is expected to increase or decrease (if the coefficient is positive or negative respectively) when that independent variable increases by one and p-value with 95 % confidence correlate both variables. As 95 % of the t-distribution is nearer to the mean than the t-value on the coefficient which shows that level of significance is 5% and it is generally accepted criteria to reject the null hypothesis. With a p-value of 5% or 0.05 there is only a 5 % chance that indicates 95% probability of being correct that the variable under studies is having certain effect. Small p-value i.e. 0.2192 has been obtained with a

Table 3: Factors and their assigned levels

Serial #	Factors	Level 1	Level 2	Level 3	Level 4	Level 5
1	Time of incubation (hrs)	24	48	72	96	120
2	pH	4.0	5.0	6.0	7.0	8.0
3	Aeration (rpm)	100	120	150	180	200
4	Temperature (°C)	45	50	55	60	65
5	Nitrogen conc. (%, w/v)	0.5	1.0	1.5	2.0	2.5
6	Carbon conc. (%, w/v)	0.5	1.0	1.5	2.0	2.5

Table 4: Optical Density (OD) of inoculum under given factors before and after incubation at 650nm by using UV-Spectrophotometer at room temperature

Serial #	Factors	Blank**	OD of inoculum before incubation	OD of inoculum after incubation	Polypeptide Production (%)
1	Time of incubation (hrs)	0.00	0.59021	1.02140	77.36
2	pH	0.00	0.60109	1.01119	69.33
3	Aeration (rpm)	0.00	0.61540	1.01257	69.07
4	Temperature (°C)	0.00	0.67018	1.05024	73.33
5	Nitrogen conc. (%, w/v)	0.00	0.70019	1.04129	73.74
6	Carbon conc. (%, w/v)	0.00	0.60799	1.00421	69.50

*Polypeptide production activity after incubation was evaluated through well diffusion assay. At high optical density, greater zone of inhibition was observed against *Staphylococcus aureus* as also shown in fig. 2.
**Blank values indicate the neutral values and considered as zero (without any change).

significant F-value i.e. 0.2191 (fig. 6). Orthodox optimization techniques proceeded by varying the single factor at one time while other factors remained constant that permits to measure the influence of those specific factors on the experimental activity. Using DOE for quantitative data obtained from an appropriate experimental design to fix and solve multivariate problems, simultaneously.

Table 5: L25 Trials (5^6) Orthogonal Arrays (OA)

	Columns and variable settings						*Percentage
	1	2	3	4	5	6	
1	1	1	1	1	1	1	54.45
2	1	2	2	2	2	2	69.33
3	1	3	3	3	3	3	73.54
4	1	4	4	4	4	4	69.29
5	1	5	5	5	5	5	53.21
6	2	1	2	3	4	5	75.35
7	2	2	3	4	5	1	73.35
8	2	3	4	5	1	2	73.22
9	2	4	5	1	2	3	77.36
10	2	5	1	2	3	4	73.53
11	3	1	3	5	2	4	69.50
12	3	2	4	1	3	5	70.71
13	3	3	5	2	4	1	50.25
14	3	4	1	3	5	2	73.53
15	3	5	2	4	1	3	55.51
16	4	1	4	2	5	3	69.07
17	4	2	5	3	1	4	70.85
18	4	3	1	4	2	5	49.97
19	4	4	2	5	3	1	73.74
20	4	5	3	1	4	2	54.50
21	5	1	5	4	3	2	73.74
22	5	2	1	5	4	3	69.33
23	5	3	2	1	5	4	69.50
24	5	4	3	2	1	5	55.89
25	5	5	4	3	2	1	71.20

***** Percentage of polypeptide production was measured by taking optical density (650nm) under UV spectrophotometer before and after incubation under given conditions and activity evaluated through well diffusion assay

The test outcomes were investigated to evaluate the core properties of the parameters; the study of standard variance procedure was then used to conclude statistically important factors. The critical aspects were sorted out with the important impact of statistical applications. As there were 5-levels for each factor (5^6), therefore, L-25 Orthogonal Array (OA) was selected for the experimental design. The average effects of the affecting parameters and their relevant interactions at the given levels on polypeptide antibiotic synthesis are shown in table 6. The significant change of each parameter at level 2 and 1 shows the qualified impact of the affecting conditions. The sign of the change (+ or -) specifies whether the modification from level 1 to 2, 3 or 4 enhanced or reduced the outcomes (table 6). Established data showed that time of incubation, nitrogen concentration, carbon concentration and aeration rate showed a positive influence on result as compare to other factors (table 6). However, when interaction of altered factors were measured in the present study (table 7), it is exciting to note that the most significant aspects including the interaction between time of incubation and nitrogen concentration revealed best severity index (SI) while other interactions such as carbon and nitrogen concentration showed least (56.67 %) severity index (table 7). These outcomes elucidated that the impact of single parameter on antibiotic synthesis was dependent on the value of the other aspects during optimizing the experiment parameters. The pure sum and percentage involvement of each factor is shown in ANOVA table (table 8). The calculation of the change for each factor and interaction trials indicated that the least values were attained for the factors including temperature and aeration rate. The last column of the ANOVA (table 8) shows the effect of each parameter. Performing ANOVA on table 8 results showed that all factors are effective on the response variables. It is necessary to discuss that we considered the confidence level of 95 % in our experimental study. So, lower p-values exhibited the effectiveness of a factor on the response variables.

Response surface methodology (RSM) was applied in experimental design and data analysis to consider two

related factors: time of incubation/nitrogen concentration, time of incubation/pH and their interaction (fig. 7). This contour plot exhibited the significant interactive parameters that affected the optimum production of polypeptide antibiotic. The optimum production and highest inhibitory effect of this compound was obtained at pH 5 with 1.5 % nitrogen concentration at 48 hours of incubation time. Point prophecy of the design indicated that 20mg/100mL of antibiotic production was attained under scale up studies.

DISCUSSION

The present study was carried out with the objective of isolation, identification, screening, and production of peptide antibacterial secondary metabolites by thermophilic bacterial strains. Bacterial secondary metabolites possessing antimicrobial or antifungal activity including peptides (Zuber and Marahiel, 1997) are getting worth now a day and due to number of clinical concerns their usage is important (Schallmey *et al*., 2004). The increase in drug resistance has restricted our selection and finding of bio active antibacterial molecule, creating a challenging situation that has been worsened by the small number of new drug molecules reported recently (Kohanski *et al*., 2010).

The strain was identified on the basis of standard, morphological, biochemical and molecular 16S rRNA methods. Morphologically, isolate was a Gram-positive rod shaped, motile and spore forming (Yasawong *et al*., 2011) while biochemical studies indicated that citrate utilization, oxidase, starch and casein hydrolysis were positive while indole, catalase and gas production tests were negative with the positive fermentation of glucose showing acid production (Kandler and Weiss, 1986; Bae *et al*., 2005). Molecular studies showed that isolate matches the *Geobacillus pallidus*. These findings determined that this *Geobacillus* specie is associated with the production of thermostable polypeptide antibacterial agent which is not reported before.

Precipitation of protein was at 50 % ammonium sulphate concentration. In 1991, Shimogki *et al*. reported that the first step in the purification of the antibiotic was separation of crude antibiotic from the microbial growth followed by precipitation of proteins by 70 % ammonium sulfate (Shimogki *et al*., 1991). A lyophilized antibacterial protein sample was subjected to polyacrylamide gel electrophoresis (12%) in the presence of sodium dodecyl sulphate (SDS) and protein bands were localized by staining with coomassie brilliant blue dye (Atta *et al*., 2009).

Though peptide antibiotics are produced by various species of actinomycetes, lower fungi, but bacteria of the genus Bacillus are the most numerous producers. Antimicrobial secondary metabolites occur in some species of thermophilic actinomycetes (Kosmachev *et al*., 1965) and fungi (Saito *et al*., 1979), but virtually nothing is known of thermophilic bacilli producing antibiotic substances, including peptides. However, thermophilic bacterial species have also been recognized but as compare to mesophilic genera, they have not been analyzed well (Edwards, 1993). Bacilli producing peptide antibiotics (gramicidins, tyrocidines, and bacitracins) are mainly mesophilic (Egorov *et al*., 1987). Few of them are capable of growing at temperatures above 40°C. They include Bacillus brevis var. G-B, producing gramicidin C (Egorov, 1999), and *Bacillus polymyxa*, producing gavaserin and saltavalin (Pichard *et al*., 1995). In 2002, Esikova *et al*. reported the thermophilic Bacillus species strains VK2 and VK21 involved in the production of peptide antibiotics.

The antibacterial metabolite production from *Geobacillus pallidus* SAT4 was optimized by considering the physical factors including the time of incubation, pH, temperature, aeration rate, and chemical factors including nitrogen, and carbon concentrations as these parameters are known to affect the cultural growth and synthesis of antimicrobial molecules (Webster *et al*., 2002). Improved approaches are needed to optimize and enhance the efficiency of presently available drugs due to the slow speed of antimicrobial drug development (Falagas and Kopterides, 2007). Antibiotic synthesis by using producing bacteria varies quantitatively and qualitatively based on the factors including the microbial strains and their growth fermentation conditions (Chen *et al*., 1996). In this study the production was optimized at different conditions by using Taguchi approach, which is a fully developed method has advantageous including saving process time, and reducing the experimental cost and is a best choice for the optimization of fermentation procedures for synthesis of microbial metabolites (Houng *et al*., 2006) to investigate the accumulative effects of the process-variables including physical (time of incubation, aeration rate, pH, temperature) and chemical factors (carbon and nitrogen concentrations).

The Taguchi method includes to reduce the variation in a process through robust design of experiments whose performance is least affected by these noises. Analysis of results leads to a response model in which the relationship of each variable (i.e., factor) towards the response, as well as the interactions between factors, is shown. In regression with a single independent variable, the coefficient exhibits how much the dependent variable is expected to increase or decrease (if the coefficient is positive or negative respectively) when that independent variable increases by one and p-value with 95 % confidence correlate both variables. As 95 % of the t-distribution is nearer to the mean than the t-value on the coefficient which shows that level of significance is 5%

Table 6: Main effects

Serial #	Factors	Level 1	Level 2	Level 3	Level 4	Level 5	L2-L1
1	Time of incubation (hrs)	24	48	72	96	120	10.598
2	pH	4.0	5.0	6.0	7.0	8.0	2.292
3	Aeration (rpm)	100	120	150	180	200	4.524
4	Temperature (°C)	45	50	55	60	65	-1.690
5	Nitrogen conc. (%, w/v)	0.5	1.0	1.5	2.0	2.5	5.488
6	Carbon conc. (%, w/v)	0.5	1.0	1.5	2.0	2.5	4.266

Table 7: Estimated interaction of severity index for different parameters

Serial #	Factors	Columns [a]	SI (%) [b]	Col [c]	Levels [d]
1	Time of incubation x Temperature	1 X 4	63.63	5	[2,3]
2	Nitrogen conc. x pH	5 X 2	60.50	7	[3,2]
3	Aeration x Temperature	3 X 4	60.31	7	[4,3]
4	Time of incubation x pH	1 X 2	67.51	3	[2,2]
5	Temperature x Carbon conc.	4 X 6	56.84	2	[3,4]
6	Nitrogen conc. x Temperature	5 X 4	57.02	1	[3,3]
7	Temperature x pH	4 X 2	60.65	6	[3,2]
8	Carbon conc. x Time of incubation	6 X 1	63.23	7	[4,2]
9	Aeration x pH	3 X 2	64.35	1	[4,2]
10	pH x Carbon conc.	2 X 6	60.27	4	[2,4]
11	Time of incubation x Aeration	1 X 3	67.13	2	[2,4]
12	Carbon conc. x Nitrogen source	6 X 5	56.67	3	[4,3]
13	Nitrogen conc. x Aeration	5 X 3	60.16	6	[3,4]
14	Aeration x Carbon conc.	3 X 6	59.93	5	[4,4]
15	Time of incubation x Nitrogen conc.	1 X 5	67.46	4	[2,3]

[a] columns represents the column positions to which the interacting factors have been assigned
[b] SI represents the severity index (10 percent for 90 angle between the lines, 0% for parallel lines)
[c] Col shows the column that should be reserved if this interaction effect were to be studied (2-L factors only)
[d] Opt Levels represents the factor levels desirable for the optimum conditions. If an interaction is included in the study and found significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum conditions without considerations of any interaction effects

Table 8: Analysis of variance (ANOVA)

Serial #	Factors of study	DOF	Sums of squares	Variance	F-ratio	Pure sum	Percent
1	Time of incubation (hrs)	4	440.286	110.072	1.403	126.45	3.663
2	pH	4	336.853	84.213	1.073	23.017	0.666
3	Aeration (rpm)	4	153.735	38.434	0.4898	0.000	0.000
4	Temperature (°C)	4	282.103	70.526	0.8988	0.000	0.000
5	Nitrogen conc. (%, w/v)	4	364.703	91.176	1.162	50.867	1.473
6	Carbon conc. (%, w/v)	4	305.325	76.331	0.9728	0.000	0.000
	Errors	20	1569.171	78.459			94.198
	Total	44	3452.176				100.00

and it is generally accepted criteria to reject the null hypothesis. With a p-value of 5% or 0.05 there is only a 5% chance that indicates 95 % probability of being correct that the variable under studies is having certain effect. Small p-value i.e. 0.2192 has been obtained with a significant F-value i.e. 0.2191 (fig. 6). Orthodox optimization techniques proceeded by varying the single factor at one time while other factors remained constant that permits to measure the influence of those specific factors on the experimental activity. These events have limitations including time loss, burdensome, need more investigational datasets and are unable to deliver the comprehensive results about the communal interactions of the factors (Beg *et al*., 2003). Other to predictable scale up processes, design of experiment (DOE) and statistical applications support to access more evidence related to these effective conditions in a limited trials (Krishna *et al*., 2005). Using DOE for quantitative data obtained from an appropriate experimental design to fix and solve multivariate problems, simultaneously. The equations and

calculations that define the effect of the test variables, factors and other parameters on the responses control interrelationships among test variables and signify the mutual effects of all test variables in the response. This approach enables us to make efficient exploration of an experimental process. These factors are constant during the process design and effect of them is equal for each experiment (Madamba, 2002). The key mechanism of this process functions as filters, which inspect the special impacts of many variables and recognize those parameters, which have main effects on method using a limited number of tests (Dasu *et al*., 2003). Taguchi design of trials includes developing the great number of investigational conditions termed as orthogonal arrays (OA) to reduce test inaccuracies and to improve their productivity and reproducibility of the laboratory experiments (Krishna *et al*., 2005).

This investigational design is a useful selection for the scale up of bioreactor and industrial level developments for enhanced yield of microbial secondary metabolites. Among several statistical investigational studies, Taguchi experimental design deals distinctive gains by which several factors can be studied concurrently and abundant quantitative data can be mined with a limited number of experimental studies (Houng *et al*., 2006; Stone and Veevers, 1994). The parameters which were optimized include time of incubation at 48 hours, pH of the medium at 5, temperature at 55°C, aeration rate at 180 rpm, nitrogen concentration at 1.5 % and carbon concentration at 2.0%. The test outcomes were investigated to evaluate the core properties of the parameters; the study of standard variance procedure was then used to conclude statistically important factors. The critical aspects were sorted out with the important impact of statistical applications. As there were 5-levels for each factor (5^6), therefore, L-25 Orthogonal Array (OA) was selected for the experimental design. The average effects of the affecting parameters and their relevant interactions at the given levels on polypeptide antibiotic synthesis are shown in table 6. The significant change of each parameter at level 2 and 1 shows the qualified impact of the affecting conditions. The sign of the change (+ or -) specifies whether the modification from level 1 to 2, 3 or 4 enhanced or reduced the outcomes (table 6). Established data showed that time of incubation, nitrogen concentration, carbon concentration and aeration rate showed a positive influence on result as compare to other factors (table 6). Venil and Lakshmanaperumalsamy (2009) used the Taguchi technique to assess the relative significance of nutritional media components such as carbon, nitrogen, minerals and physical factors for instance temperature and agitation speed in enzyme 'protease' synthesis by *Bacillus subtilis* strain HB04. However, when interaction of altered factors were measured in the present study (table 7), it is exciting to note that the most significant aspects including the interaction between time of incubation and nitrogen concentration revealed best severity index (SI) while other interactions such as carbon and nitrogen concentration showed least (56.67 %) severity index (table 7). These outcomes elucidated that the impact of single parameter on antibiotic synthesis was dependent on the value of the other aspects during optimizing the experiment parameters. The pure sum and percentage involvement of each factor is shown in ANOVA table (table 8). The calculation of the change for each factor and interaction trials indicated that the least values were attained for the factors including temperature and aeration rate. The last column of the ANOVA (table 8) shows the effect of each parameter. Performing ANOVA on table 8 results showed that all factors are effective on the response variables. It is necessary to discuss that we considered the confidence level of 95% in our experimental study. So, lower p-values exhibited the effectiveness of a factor on the response variables.

As Venil and Lakshmanaperumalsamy reported (2009), we have also found in our study that thermostable protein antibiotic production by *Geobacillus* SAT4 in submerged culture fermentation, the glucose (2 %) as carbon source, glutamic acid (1.5 %) as nitrogen source, temperature (55^oC), pH (5), time of incubation (48 hrs) and agitation (150 rpm) up to level 5 are the important conditions of the secondary medium. The involvement of 5-factors in antibiotic synthesis using the Taguchi approach showed that time of incubation, nitrogen concentration and pH contributed important role than any other particular factors. Response surface methodology (RSM) was applied in experimental design and data analysis to consider two related factors: time of incubation/nitrogen concentration, time of incubation/pH and their interaction (fig. 7). This contour plot exhibited the significant interactive parameters that affected the optimum production of polypeptide antibiotic. The optimum production and highest inhibitory effect of this compound was obtained at pH 5 with 1.5% nitrogen concentration at 48 hours of incubation time. Point prophecy of the design indicated that 20mg/100mL of antibiotic production was attained under scale up studies. Such outcomes would further facilitate and enable the cheap and economic procedures of the industrial scale fermentation operation.

CONCLUSION

Using Taguchi's methodology, we have recognized critical variables and interactions that could be applied for further experimentation intended at optimizing the production of peptide antibiotics at shake flask level fermentation. It was concluded that novel thermostable protein was produced by the *Geobacillus pallidus* strain SAT4, is stable at higher temperature.

There were two most significant aspects that affect the

synthesis of antibiotic including time of incubation and nitrogen concentration and two interactions including the time of incubation/pH and time of incubation/nitrogen concentration were recognized as being the vital aspects that affect the polypeptide production.

This interpretation at lab scale level experimentation can be used as a foundation for optimization of antibiotics at reactor scale level. This study will be helpful to find out and determination of deviations and shows the great value of using the Taguchi's technique to easily define the critical parameters to be optimized for optimum production.

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