International Journal of Psychosocial Rehabilitation, Vol. 24, Issue 08, 2020
ISSN: 1475-7192

Optimization Production of Synthetic Recombiant Analgesic Peptide Molecules in Yeast by using Taguchi Methodology.

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Abstract--*The analgesic peptide synthetic genes with signal peptides were sub cloned into the pCTON2 for expression in yeast. The recombinant plasmids were used to transform under the gal promoter of yeast. The L-8 orthogonal array was selected for screening of the factors and interaction between seven factors selected: size of inoculum, temperature, orbital speed, time of induction, concentration of antibiotics, volume of container and media at two levels. After screening, more effective factors and their interactions were optimized using L8 and experimental configuration of orthogonal arrays. Qualitek-4 software was used for automatic design and standard ANOVA method of Taguchi experiments. ANOVA has revealed and identified the effect of each factor and the optimum conditions and estimated the performance of the each condition was evaluated. Currently, the achievable volumetric productivities for yeast cultivations are reaching about 1-2 mg/L/h, which exceed that of most of the mammalian cell productivities (~0.5–1.0 mg/L/h). The expected Taguchi results indicated soluble analgesic peptides expression level of 48 mg/l under optimal conditions can be achievable in shake flask cultures by using Taguchi Method. The same value o of 45 mg was obtained experimentally for analgesic opioid peptide expression under optimum conditions in shake flasks. pH, RPM and size of the inoculums have been identified as crucial factors as per the optimization and statistically suggested methods and rest of the factors are kept at normal conditions.*

Key Words--*Analgesic Peptides, pCTON2 Plasmids, Yeast Expression, Taguchi Methodology, Qualitek-4, ANOVA.*

I. INTRODUCTION

Advances in both Genetic Engineering and Pharmacological Sciences for quite some time made it possible for us to explore for more potent neuropeptides which can play a significant role in understanding the transmission of pain impulses and their signal transduction cascades in various neurological conditions such as pain reflexes. These neuropeptides which are synthesized in laboratory are working to match with targeted cell membrane receptors in specific locations in a well defined manner.

Even though Peptide Chemists have developed small molecules like Biphalin, Tachykinin but due to their high cost productions and much less stability of these molecules they are not successfully competing in the clinical trials. These gaps have created an opportunity for rDNA technologists to develop novel therapeutic analgesic peptide molecules which are small in size, high in stability and roubst in action and with showing less side effects [1].

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DOI: 10.37200/IJPR/V24I8/PR280159
Received: 21 Jan 2020 | Revised: 08 Feb 2020 | Accepted: 14 Mar 2020

Hence Biotechnology products using rDNA Technology will give us an ample of opportunity to prepare and produce these small neuro peptide molecules on large scale production for industrial applications [2]. Hence the current work emphasizes on production of these novel analgesic neuro peptide molecules designed, and synthesized by protein engineering methods and expressed in Eukaryotic organisms like Yeast Cells for highest level production capabilities.

Optimum conditions are required for economical and high-yield production in chemical engineering and biotechnological processes, therefore the optimization of medium components and operational variables are of vital importance to the fermentation process [3]. Statistical methods plays crucial in improvement of productivity levels because they play an important role in experimental design, evaluation of factors and optimization of medium composition and operational variables. Several statistical methods are widely used in biological processes [4-7]. Among them, the Taguchi method serves as a screening filter which examines the effects of process variables and identifies those factors that have major contributory role on the process through the use of a single trial with a few experiments [8-10]. Hence, current work is carried out to find optimzable parameters by using Taguchi method and successfully implemented for high production of analgesic peptide molecules in shake flask culture method.

II. MATERIAL AND METHODS

Cloning of Analgesic sequences in Sacchromyces cerviseae

Codon optimization has been carried out for maximum expression in yeast and E.coli. The designed specific synthetic genes were synthesized at Gene Art, Germany. The analgesic peptides were cloned in yeast expression vector pCTON2 and transformed in EBY100 yeast cells for expression.

Beta-endorphin: AA Sequence: YGGFMTSEKSQTPLVTL FKNAIIK NAYKKGE

CodonoptimizationSeq:TATGGTGGTTTTATGACTTCTGAAAAATCTCAAACTCCACTGGTTACTCTGT TTAAAAATGCTATTATTAAAAATGCTTATAAAAAAGGTG AA

Plasmid construction

The analgesic peptide synthetic genes with signal peptides were subcloned into the pET 22b vector for bacteria and pCTON2 for yeast (Novagen). The recombinant plasmids were used to transform under gal promoter in yeast.

Cloning of wild and Mutein sequences in suitable host organisms for maximum expression and activity

The selected mutant sequences of peptides were codon optimized using DNA 2.0 software for maximum expression in *Saccharomyces cerevisiae and E.coli*. Codon optimization was carried out at above 15% threshold and as per the codon usage frequency of standard *Saccharomyces and E.coli* tables. For yeast AlphaF mating tag and for E Coli ompA tag was used for extracellular expression.

Secretary Signals in Yeast is alpha F with aminoacid sequences of MRFPSIFTAVLFAASSALAAPVNT TTT AQIPAE AVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKR.

DOI: 10.37200/IJPR/V24I8/PR280159
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Optimization of recombinant peptide production in Yeast:

Compared with mammalian cells, the main advantages of yeast production are that it requires only cheap, defined media and an ability to generate high cell densities up to 100 g dry cell/L in short cultivation times.

Qualitek-4 Software for Automatic Design and analysis of Taguchi Experimemts:

Optimum conditions are the basis for high economical and greater-yield production in chemical engineering and biotechnological processes; therefore the optimization of medium components and operational variables are of vital importance to the fermentation process. Statistical methods are crucial for improvement of productivity because they play an important role in experimental design, evaluation of factors and optimization of medium composition and operational variables. Several statistical methods are widely used in biological processes. Among these, the Taguchi method serves as a screening filter which examines the effects of process variables and identifies those factors that have major effects on the process through the use of a single trial with a few experiments. The following optimization parameters/operational variables used for Taguchi statistical method at two levels in shake flask culture.

Table 1: Parameters Selected in Optimization of Cultures by Taguchi Method.

S.NO	Effecting Factors on Yeast Culture	Level -1	Level-2
1	pH	7.4	6.8
2	Temperature in Degrees	30	25
3	Shaking RPM	150	100
4	Induction Period in Hrs	12	6
5	Size of inoculums in ml	10%	5%
6	Volume of Media in ml	100	100
7	Volume of Container in ml	300	250

In order to maximize protein expression in shake flask culture, the above selected parameters and the effect of operational variables (s*ize of inoculum*, temperature, rpm, time of induction, pH, volume of container and media) and also their significant interactions were optimized by using the Taguchi statistical method. The L-8 orthogonal array was selected for screening of the above factors and interaction between seven factors: *size of inoculum*, temperature, orbital speed, time of induction, concentration of antibiotics, volume of container and media at two levels. After screening, more effective factors and their interactions were optimized using L8 and experimental configuration orthogonal arrays.

Qualitek-4 software was used for automatic design and standard ANOVA of Taguchi experiments. ANOVA revealed and identified the effect of each factor and the optimum condition and estimated the performance of the optimum condition.

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Taguchi experimental design for screening of selected factors

In order to optimize the operational conditions for the rapid growth of yeast and maximum expression of recombinant analgesic peptides in shake flask culture, the Taguchi statistical design was applied. Firstly, the standard L8 orthogonal array was used for the screening of seven variables containing seven working factor interactions were carried out.

Column # / Factors	Level 1	Level 2	L2 - L1
1 pH	38.723	43.366	4.643
2 Temperature	38.096	43.993	5.897
3 Agitation (RPM)	38.646	43.443	4.796
4 Inoculum size	40.91	41.179	.269
5 Anti biotic conc	41.501	40.588	-.913
6 Flask volume	42.824	39.265	-3.559
7 Inducer	41.905	40.184	-1.722

Figure 1. Average effect of Factors and Interactions.

Total Seven factors were selected for optimization studies i.e., pH, Temperature, Agitation RPM, Inoculum size, Antibodies Concentration, Flaks volumes and Inducers and their level of effects were observed. Majorly the big differences between Level1 to Level2 are observed between Temperature (5.8), Agitation (4.7), pH (4.6), Inoculum size (0.26), Antibiotic Concentration (0.91), Inducer (0.17) and Flask Volume (0.35). These parameters has to optimized to get maximum yields for the analgesic peptides.

Presence of the interaction has been selected for among all factors and has been studied in the following manner and the severity indexes among the factors were noted and given for analysis by the Taguchi Software using the following matrix table with 21 combinations to get the best optimization for peptide yields.

Number of interactions between two factors calculated = 21

#	Interacting Factor Pairs (Order based on SI)	Columns	SI(%)	Col	Opt.
1	Vol. of Media in ml x Vol. of Cantainer in ml	6 x 7	79.26	1	[1,2]
2	Temperature in degrees x Vol. of Cantainer in	2 x 7	74.28	5	[1,2]
3	Incubation period in hrs x Vol. of Cantainer in	4 x 7	70.12	3	[2,1]
4	Temperature in degrees x Vol. of Media in ml	2 x 6	64.88	4	[1,2]
5	pH x Temperature in degrees	1 x 2	62.91	3	[2,1]
6	Size of inoculum in ul x Vol. of Media in ml	5 x 6	60.02	3	[2,1]
7	pH x Incubation period in hrs	1 x 4	53.04	5	[2,1]
8	Incubation period in hrs x Size of inoculum in ul	4 x 5	46.95	1	[1,2]
9	Orbital speed in rpm x Vol. of Media in ml	3 x 6	39.97	5	[2,1]
10	Temperature in degrees x Orbital speed in rpm	2 x 3	37.08	1	[1,2]
11	Incubation period in hrs x Vol. of Media in ml	4 x 6	35.11	2	[2,2]

#	Interacting Factor Pairs (Order based on SI)	Columns	SI(%)	Col	Opt.
11	Incubation period in hrs x Vol. of Media in ml	4 x 6	35.11	2	[2,2]
12	pH x Size of inoculum in ul	1 x 5	33.93	4	[2,2]
13	Orbital speed in rpm x Vol. of Cantainer in ml	3 x 7	29.87	4	[2,1]
14	Size of inoculum in ul x Vol. of Cantainer in ml	5 x 7	25.71	2	[2,2]
15	Temperature in degrees x Incubation period in	2 x 4	23.48	6	[1,2]
16	pH x Vol. of Cantainer in ml	1 x 7	20.73	6	[2,2]
17	pH x Orbital speed in rpm	1 x 3	14.5	2	[2,2]
18	Orbital speed in rpm x Size of inoculum in ul	3 x 5	9.25	6	[2,2]
19	pH x Vol. of Media in ml	1 x 6	9.01	7	[2,1]
20	Temperature in degrees x Size of inoculum in ul	2 x 5	7.47	7	[1,2]
21	Orbital speed in rpm x Incubation period in hrs	3 x 4	4.7	7	[2,2]

Fig 2: Interaction of Factors at Different Levels.

The values are summerized in the following table 2. The severity indexes vs interaction between factors are listed.

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Table 2: Interactions of Factors with Severity Indices:

Interaction between Factors	Severity Index
pH and Shaking Speed	14.5
Temp and Incubation period	23.5
Size of Inoculum and Volume	25.7
RPM and Volume of Container	29.9
pH and Size of Inoculum	33.9
Incubation period and Volume	35.1
Temp and Shaking Speed	37.08
Volume of media and Shaking	39.97
Incubation Period and Size of	47
pH and Incubation period	53.04
Size of Inoculum and Volume	60.02
pH and Temperature	62.9
Temp and Volume of Media	64.9
Incubation period and Vol. of	70.1
Temperature and Volume	74.3

The interactions between pH and Shaking have the severity index of 14.5. The interactions between pH and volume of container have the severity Index of 20.7. The interactions between temperature and incubation period have the severity index of 23.5. The interaction between size of inoculum and volume of container have the severity index of 25.7. The interactions between RPM and volume of container have the severity index of 33.9. The interactions between incubation period and volume of media have the severity index of 35.1. The interaction between temparature and shaking speed have the severity index of 37.08. The interactions between volume of media and shaking speed have the severity index of 39.97. The interactions between incubation period and size of inoculum have the severity index of 47.0. The interactions between pH and incubation period have the severity index of 53.04. This may be due to the secretion of metabolite This indicates that there should be sufficient use of inoculums for particular volumes of media used. The interactions between pH and Temperature have the severity index of 62.9. Temperature plays an important role in the growth of the culture and active production of peptides with active form. The interactions between incubation period and vol.of container have the severity index of 70.1. To get sufficient growth of culture and products need necessary time of incubation along with the volume of container used. The interactions between temperature and volume of container have the severity index of 74.3. The interactions between volume of media and volume of container have the highest severity index of 79.3. and accumulation. The interaction between size of inoculums and volume of severity index of 60.2.

The main factors and their effects on the growth and production of recombinants have been shown the forms of normal plots along with their contribution at two levels were presented as multiples plots.

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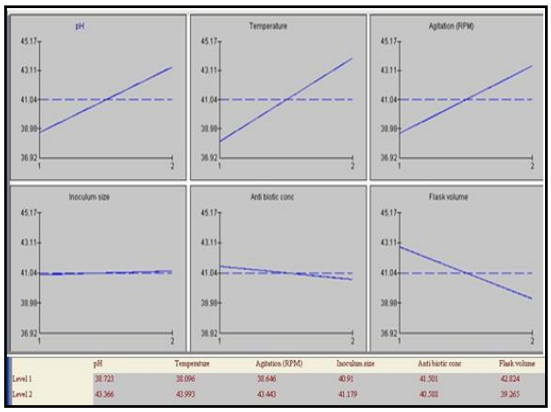

Fig 3: Effect of Various factors on Analgesic Peptides production.

Results were then analyzed by standard ANOVA for determination of significant variables to find the optimized conditions. This shows that shaking speed and size of inoculums have maximum contribution and then pH on production of analgesic peptides.

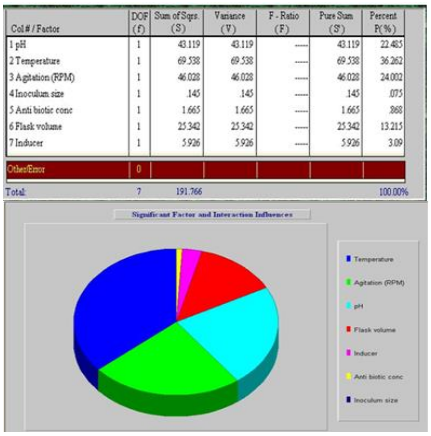

Fig 4: Relative influence of factors on Analgesic production

Shaking speed, size of the inoculums, pH and volume of the media has shown the significant influence on the growth of the recombinants. The data were analysed by standard ANOVA, and the percentage contribution of each variable and the optimum level were then obtained as given below

Optimized conditions for yeast

According to the Taguchi statistical analysis for the cultivation of yeast has been optimized and the following parameters have been chosen obtained. Observations and results of the experiments were discussed in the results section.

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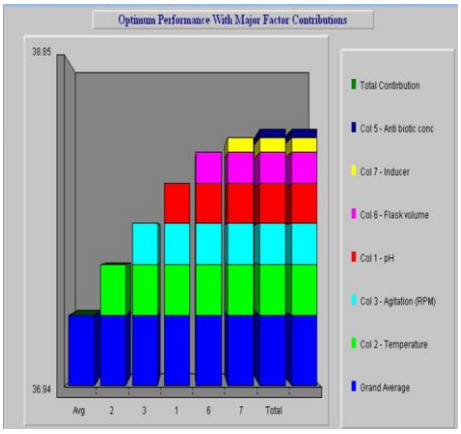

Fig 5: Optimum Condition and Performance.

Testing and Evaluation of Parameters

Performance distribution between control and optimized conditions were plotted on variation reduction plot to find the improvement in the distribution of the results. Above normal distribution graphs shows the satisfactory improvement in the average values 67+3.5 when the target value is 70.

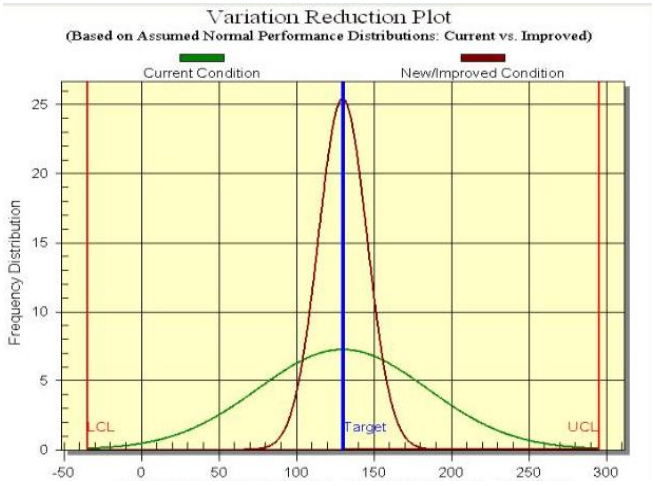

Figure 6: Factor contribution stacked graph

Verification of Taguchi optimized results

Currently, the achievable volumetric productivities by yeast cultivations are about 1-2 mg/L/h, which exceed that of most of the mammalian cell productivities (~0.5–1.0 mg/L/h). The expected Taguchi results indicated soluble analgesic peptides expression level of 48 mg/l under optimal conditions in shake flask culture. A value of 45 mg was obtained experimentally for analgesic opioid peptide expression under optimum conditions in shake flask. pH, RPM and size of the inoculums have been modified as per the optimization by statistically suggested and rest of the factors are kept at normal condition.

III. CONCLUSION

The work presented here is a first report that has used the Taguchi statistical design method for the expression and production in recombinant pCTON2 for yeast. These results, derived at the laboratory scale, provides an improved set of conditions and a basis for further investigations involving the larger-scale production of recombinant pCTON2 for yeast by the high-cell-density cultivation method.

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Received: 21 Jan 2020 | Revised: 08 Feb 2020 | Accepted: 14 Mar 2020

Acknowledgement

The author is highly thankful for the financial support under DBT-RGYI scheme to Govt of India.

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