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Optimization of media components for the production of *N*-acetylchitooligosaccharide from chitin by *Streptomyces chilikensis* through Taguchi experimental design

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ABSTRACT

Optimization of media composition for microbial growth is crucial particularly in industrial processes to obtain the desired end product. The waste from sea food industries includes the non-edible parts of shrimp, crabs and prawns which are rich in chitin as the major cause of pollution in coastal areas. Chitin degradation is carried out chemically. It can be degraded biologically also, particularly using microorganisms resulting in chitooligosaccahrides and the monomer *N*-acetylglucosamine. *N*-acetyl glucosamine and related chitooligosaccahrides have various applications such as treatment of cancer and metastasis, treatment of autoimmune reactions, as food supplements and increased plant stress tolerance against salinity and heavy metals. Thus, chitin waste can be efficiently degraded biologically using microorganisms to produce such useful products. Conventional methods such as One factor at a time (OFAT) are more time consuming and costly to address the problem. The current work focuses on the development of an experimental design to ascertain parameters optimized for chitin degradation by a *Streptomyces chilikensis* to produce various chitooligosaccharides. More than one factor was taken at a time to carry out the experiments and the data were fit into Taguchi Design to determine the contribution of the most important factors responsible for the production of the desired end product that is NAG and other chitooligosaccaharides. Highest NAG production (3741 μM/reaction) was observed in a media that contains 0.5% Raffinose (w/v), 0.5% peptone (w/v), 2.5% NaCl at pH 11.

1. Introduction

Chitin, the second most abundant biopolymer in the world (Shahidi and Abuzaytoun, 2005) is an insoluble linear polymer of β (1–4) linked N acetyl D glucosamine. It is widely distributed in nature as a structural component of crustaceans, fungi, protozoa, insects, crabs. (Flach et al., 1992). Chitin occurs in three polymorphic forms such as α, β, and ϒ based on the source (Campana-Filho et al., 2007). Exoskeleton of crustaceans, particularly shrimp and crabs contain mostly α chitin where as squid pens contain mostly β chitin. Higher organisms such as fungi and yeast mostly contain gamma- chitin. (Flach et al., 1992). Waste from the sea food industries including mainly the non-edible parts of shrimp, crabs, prawns that are rich in chitin have become the major cause of environmental pollution in coastal areas. Efficient utilization of the waste rich in chitin has an important role in maintaining a clean environment and also obtaining many value added biomolecules such as chitooligosaccharide and *N*-acetylglucosamine. Thus, development of an optimized method to obtain such biomolecules from chitin can be proposed in this context.

N-acetyl glucosamine is known for its antitumor, anti-hypersensitive activities. Also, it has been reported for treatment of osteoarthritis, gastritis and as a food supplement (Kirkham and Samarasinghe, 2009; Aam et al., 2010). Similarly, chitooligosacchrides are proposed as drugs against asthma, in components of wound dressings, role in reduction of metastasis and effective against osteoporosis (Aam et al., 2010). This chitin can be degraded chemically to derive these products such as chitosan, chitooligosacchrides and *N*-acetyl glucosamine. However, there are several disadvantages such as low yield and high cost of production (Chen et al., 2010). Recently enzymatic methods have also reported in this context (Kim and Rajapakse, 2005). However, the major limitations in this regard are intensive shell pretreatment processes (Aye and Stevens, 2004). In this context extracellular microbial

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chitinase showed potential application in the bioconversion of chitin loaded sea food waste to produce *N*-acetyl chitooligosaccharides (Suresh, 2012). However, use of enzymes for management of huge chitinous waste has limitations as they are costly. Interestingly > 10^{11} tons of chitin is produced annually in aquatic habitats but there is no evident accumulation found in the aquatic sediment (Souza et al., 2011) as they are efficiently degraded by the chitinolytic microorganism of the aquatic habitat. In this context use of novel chitinolytic microorganisms for the production of mentioned biomolecules by using chitin of sea food waste are being investigated.

Isolation and identification of chitin degrading enzymes has been reported by many investigators (Elieh-Ali-Komi and Hamblin, 2016; Dahiya et al., 2006.; Hoell et al., 2010). However, information regarding optimization of the process parameters in chitin degradation by the microorganism was scanty. Thus, in this study, *Streptomyces chilikensis* RC1830^T, a chitinolytic microorganism isolated from Chilika lake, Odisha, India (Ray et al., 2013) was used to investigate chitin degradation.

Efficiency of various bioprocesses involving microorganisms are dependent on several factors such as media composition and other culture conditions which play a significant role. The optimization of media composition is a great challenge because of the large number of variables. Conventional methods such as One factor at a time (OFAT) are more time consuming and costly to address the problem. To overcome this problem, an efficient statistical method was developed here that will allow the determination of appropriate operating values (Vaidya et al., 2003).

Various reports are available about the application of Taguchi Design of Experiments. (Mishra and Thakur, 2010; Mnif et al., 2014; Athreya and Venkatesh, 2012; Jahanshahi et al., 2008; Gross et al., 2014; Kaushik and Thakur, 2009; Vijayaraj and Varatharajulu, 2015; Asadi and Norouzbeigi, 2017). The basic principle of Taguchi method is to determine the contribution of important process parameters to the system under study and thus to screen the most important factors responsible for the desired output. It involves a large number of experimental combinations represented as Orthogonal Arrays (OA) to decrease the experimental errors and to augment the reproducibility of the laboratory experiments (Oskouie et al., 2007). Precisely Orthogonal arrays are plans of multifactor experiments where the columns correspond to the factors, the entries in the columns correspond to the test levels of the factors and the rows correspond to the test runs (Kacker et al., 1991).

In the present study the effects and contributions of different control parameters on the production of NAG by *S. chlikensis* RC1830T was investigated by batch culture using Taguchi experimental design with L9 orthogonal array. The study is primarily aimed at statistical optimization of various controllable factors like pH, temperature and NaCl concentration for maximizing the degradation of chitin evident by *N*-acetylated Chitooligosaccharides. The percentage contribution of each parameter is analyzed by adopting an Analysis of Variance (ANOVA) approach. A confirmation experiment was also carried out at the optimized conditions.

2. Materials and methods

2.1. Chemicals, glassware and reagents

All materials and glassware (DURAN, Wertheim, Germany) were washed and rinsed with MilliQ (RiOs 16 Century, Millipore, USA) water. The analytical grade reagents used for the chemical analyses and extraction were of pure analytical-grade and purchased from Merck chemicals (Merck Millipore, Darmstadt, Germany) and Himedia (Himedia Labs, Mumbai, India).

2.2. Preparation of colloidal chitin media

The colloidal chitin was prepared according to the method of Hsu and Lockwood (1975). Precisely, 5 g of chitin flakes were acidified with 60 ml of conc. HCl (12 N) for 1–2 h. 2 l of ice cold distilled water was added to this acidified chitin slurry till a white precipitate was obtained, that was then filtered through filter paper. After filtration, the filtered chitin precipitate was washed with ice cold water several times until the pH of the suspension was 5. The suspension was then filtered and dried in a hot air oven at 50–60 °C. Dried chitin precipitate was weighed and suspended in distilled water to make 4–5% (w/v) of colloidal solution and autoclaved at 121 °C for 15 min. The solution after sterilization was preserved at room temperature (RT) for several months and used as chitin supplement in growth media.

2.3. Microorganisms, culture conditions and production medium

2.3.1. Maintenance of cultures and production medium

S. chilikensis RC1830 was grown in Luria Bertani media supplemented with sea water (5 g/l Tryptone, 2.4 g/l Yeast extract, 2.4 g/l NaCl, sea water 500 ml/l) which was used as pre-culture for the optimization experiments. Minimal Salt Medium (Hsu and Lockwood, 1975) (K_2HPO_4: 0.7 g/l, KH_2PO_4: 0.35 g/l, $MgSO_4$: 0.5 g/l, $MnCl_2$: 0.01 g/l, $FeSO_4$: 0.001 g/l, $ZnSO_4$: 0.001 g/l, NaCl: 0.5 g/l with 1% colloidal chitin (v/v) was used as the production medium.

2.4. Standard curve for quantification of activity

The standard curve of N-Acetyl D-Glucosamine (NAG) was prepared according to Reissig et al., 1955. Precisely 1 mg/ml of NAG stock solution was prepared in distilled water. Concentration range of 50 μM to 4000 μM was selected (final volume made to 1000 μl). 0.8 M potassium tetra borate was added and boiled at 95 °C. *p*-Dimethylaminobenzaldehyde (DMAB) reagent was added and absorbance was recorded at 585 nm. The standard curve was constructed and the amount of NAG production at different experimental setups was extrapolated from the standard curve.

2.5. Evaluation of the effect of pH, carbon source, nitrogen source and salt concentration (NaCl % w/v) on NAG production

2.5.1. Effect of pH on Chitin degradation and production of NAG

Optimum pH for the culture condition was determined by evaluating five pH values (pH -5, 7, 9, 11 and 13). Five flasks containing production medium (100 ml each) (refer Section 2.4) were prepared in 250 ml Erlenmeyer flasks to study the effect of pH, inoculated with 1% (w/v) of culture and incubated at 37 °C in a shaker incubator (Innova New Brunswick, Germany).

Effect of different carbon sources on NAG production were determined by inoculation 1% (w/v) of pre culture to 100 ml each of production medium supplemented with carbon sources (0.5% w/v) glucose, galactose, arabinose, ribose and raffinose individually.

Effect of different Nitrogen sources on NAG production were determined by inoculating 1% (w/v) of pre-culture to 100 ml each of production medium supplemented with different nitrogen sources such as urea, ammonia chloride, peptone, yeast extract, tryptone, casein and incubated at 37 °C in a shaker incubator (Innova New Brunswick, Germany).

The effect of salt (NaCl) concentration (% w/v) on the production of NAG was studied in different concentrations of NaCl such as 1%, 1.5%, 2%, 2.5% and 3% (w/v) in 100 ml of production medium inoculated with 1% (w/v) of inoculum and incubated at 37 °C in a shaker incubator (Innova New Brunswick, Germany).

Culture aliquots were collected at different time intervals (12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 96 h and 120 h). The culture supernatant was collected after centrifugation at 4732 xg for 10 min. The

Table 1
Factors and their levels used in Taguchi's orthogonal array design for NAG production by RC1830.

Factors	Level 1	Level 2	Level 3
pH (A)	7	9	11
Raffinose (B)	0.1%	0.5%	1%
NaCl (C)	1.5%	2.5%	3.5%
Nitrogen (D)	0.1%	0.5%	1%

supernatant was then checked for the presence of *N*-acetylated chitooligosaccharides by a standard protocol (Reissig et al., 1955).

2.6. Design of experiment (DOE)

2.6.1. Taguchi DOE methodology

Using the Taguchi methodology, four factors such as pH, carbon Source, nitrogen source and salt (NaCl) concentration, were studied at three levels (Table 1). The three levels included 0.1%, 0.5%, 1%(w/v) of optimized carbon source, 0.1%, 0.5%, 1% (w/v) of optimized nitrogen source, 1.5%, 2.5%, 3.5% (w/v) of NaCl concentration, and three pH values such as. pH 7, 9 and 11. According to Taguchi's Orthogonal Array (OA), a total nine experiments were conducted with a layout of L_9 $(3)^4$ having 8 degrees of freedom. Here L represents the Latin square model and the subscript 9 represents the total number of experimental runs. The matrix layout of L_9 $(3)^4$ Taguchi orthogonal array design with three levels of the four factors involved in each of the nine experiments is represented in the Table 2. The experiment was conducted following the method described elsewhere in the current manuscript (Section 2.6.1).

2.6.2. Analysis of Taguchi orthogonal array methodology

In this Taguchi Experimental design analysis, all the 9 experimental runs were conducted by calculating Signal-to-Noise ratio (S/N) ratio. This (S/N) ratio helps in determining the influence of a particular parameter for the production of a desired product. It also measures the robustness of the control factors that reduce variability in a product or a process. In context of the current work, taking "Larger is better" objective, (S/N) ratio was calculated by using the following formula;

$$S/N = -10^{*} \log (\Sigma (1/Y^2)/n) \quad (1)$$

Here "Y" is the signal (amount of NAG produced) and "n" is the number of repetitions in each experiment.

This method helps in determining the percentage of contribution and relative importance of different factors [pH, carbon source, salt (NaCl) concentration and nitrogen source] in improving the product yield. The percentage contributed of several parameters and their dominance were determined. The optimal conditions for chitin degradation were then determined by combining the factors with their corresponding levels that have the highest main effect values. All the calculations were performed using Minitab 17 software (Minitab, 2014 Minitab Inc, USA).

Table 2
Matrix layout for L_9 $(3)^4$ Orthogonal array experimental design.

Trials	pH	Raffinose	NaCl	Nitrogen
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

2.6.3. Determination of optimum levels (for 4 factors pH, NaCl concentration, carbon source, nitrogen source) and validation of the Taguchi design of the experiment by unpaired t-test

After analyzing the results, the optimum levels of the important parameters for the highest NAG production were determined. The optimum conditions recommended by the software were validated by the assay method for NAG in triplicates with optimum conditions. Unpaired *t*-test was performed to check statistical significance of the model. Graph pad Prism version 7 was used for the unpaired *t*-test.

3. Results

3.1. Screening of optimum pH, Carbon Source, Nitrogen source and salt (NaCl) concentration (% w/v)

Screening for optimum parameters for chitin degradation and production of N-Acetyl chitooligosaccahride by the strain *S. chlikensis* RC1830 was carried out by the conventional "One Factor at a Time" method.

3.1.1. Effect of different pH on chitin degradation

The effect of pH on chitin degradation was studied by examining five pH values (5, 7, 9, 11 and 13) till 120 h. The N-Acetyl chitooligosaccahride production (Chitin degradation product) was observed at a pH range from 7 to 11 at 72 h. At pH 11 maximum N-Acetyl chitooligosaccahride concentration was observed while it was decreasing at pH 7 (Fig. 1).

3.1.2. Effect of carbon source on chitin degradation

The effect of different external carbon sources was studied and it was observed that the presence of 0.5% raffinose (w/v) showed maximum N-Acetyl chitooligosaccahride production as compared to Control (Absence of any external carbon source) after an incubation period 72 h followed by glucose, ribose, galactose and arabinose. The product formation was increased after an incubation period of 24 h. Maximum production was at 72 h in the presence of raffinose (Fig. 2).

3.1.3. Effect of nitrogen source on chitin degradation

The effect of different Nitrogen sources (Ammonium Chloride, Urea, Peptone, Tryptone, Casein, Yeast Extract) was studied on Chitin degradation and it was observed that the presence of 0.5% peptone (w/v) in the production medium showed maximum N-Acetyl chitooligosaccahride production after an incubation period 72 h followed by yeast extract, casein, tryptone and ammonium chloride. The presence of 0.5% Urea (w/v) showed an inhibitory effect on the Chitin degradation (Fig. 3).

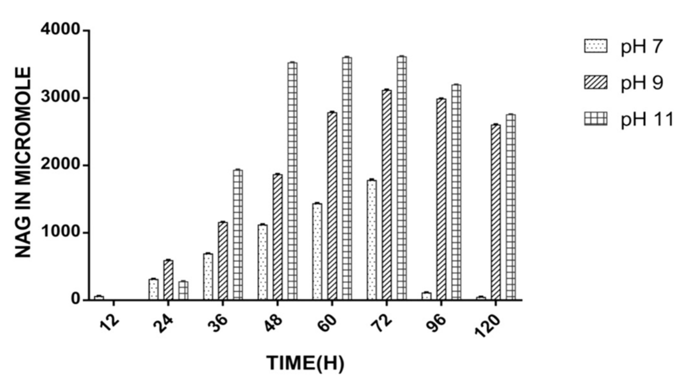

Fig. 1. Time course production of NAG by RC1830 at pH 7, 9 and 11. RC1830 was grown in production medium with test pH conditions, supernatant was collected at time intervals (12 h – 120 h) and was analyzed for NAG production. The values were plotted to decide the optimum pH.

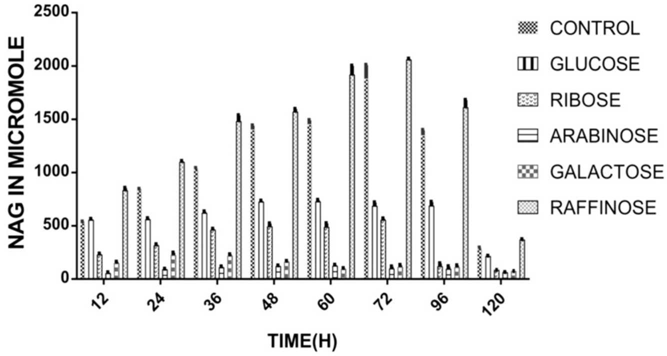

Fig. 2. Time course production of NAG by RC1830 in presence of external carbon source, supernatant was collected at time intervals (12 h–120 h) and was analyzed for NAG production. The values were plotted to determine the best Carbon source.

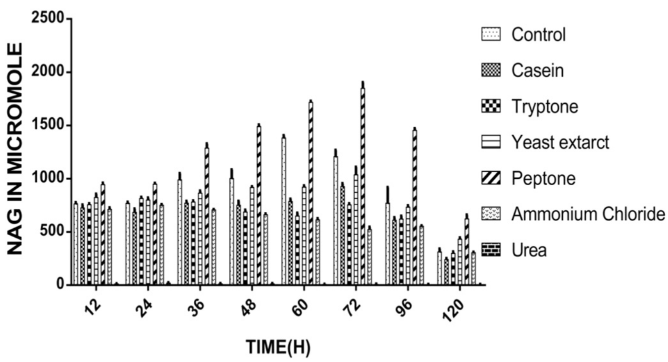

Fig. 3. Time course production of NAG by RC1830 in the presence of external Nitrogen source, supernatant was collected at time intervals (12 h–120 h) and was analyzed for NAG production. The values were plotted to determine the best Nitrogen source.

3.1.4. *Effect of different NaCl concentration on chitin degradation*

The effect of the NaCl concentration on chitin degradation and eventual N-Acetyl chitooligosaccahride formation was studied. It was observed that 2.5% NaCl (w/v) was most effective for NAG yield after an incubation period of 72 h of followed by 3%, 3.5%, 2% and 1.5% (Fig. 4).

3.2. *Results of Taguchi DOE*

In the current study of optimizing media components, the optimum values of four screened factors such as. pH (7, 9, 11), carbon source

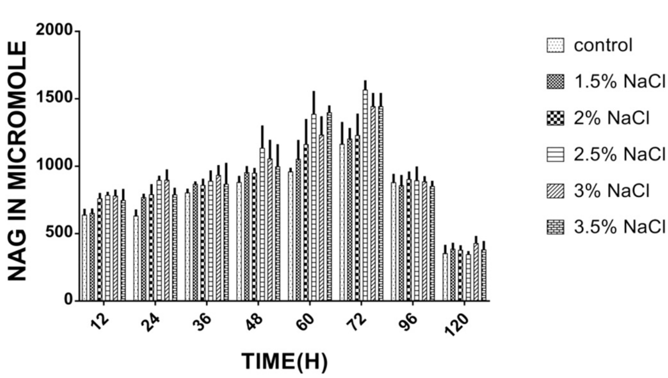

Fig. 4. Time course of NAG production by RC1830 in the presence of different NaCl concentrations. Supernatant was collected at time intervals (12 h–120 h) and was analyzed for NAG production. The values were plotted to determine the best NaCl concentration.

Table 3
Taguchi's matrix layout of the L_9 $(3)^4$ orthogonal array with response values.[a]

pH (A)	Raffinose % (w/v) (B)	NaCl (% w/v) (C)	Peptone (% w/v) (D)	Response in micromole	SNRA1
7	0.1	1.5	0.1	1799	65.10062
7	0.5	2.5	0.5	2009	66.0596
7	1	3.5	1	1800	65.10545
9	0.1	2.5	1	2731	68.72643
9	0.5	3.5	0.1	3004	69.554
9	1	1.5	0.5	3010	69.57133
11	0.1	3.5	0.5	3634	71.2077
11	0.5	1.5	1	3730	71.43418
11	1	2.5	0.1	3741	71.45975

[a] SNRA1:Signal /Noise ratio calculated using Minitab 17 software (Minitab, 2014 Minitab Inc, USA).

(Raffinose), nitrogen source (Peptone) and salt conc (NaCl (2.5%) (w/v) were determined using Taguchi Orthogonal Array design. These optimum values were evaluated by nine experimental runs. For all nine Taguchi experimental runs culture supernatants were collected at eight time points i.e. 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 96 h and 120 h. The results showed that N-Acetyl chitooligosaccahride production was maximum in experimental run 9 at 72 h, pH 11, 0.5% raffinose (w/v), 1.5% NaCl (w/v), 1% peptone (w/v) (Table 3). The NAG production was low in experimental run 1 with pH 7, 0.1% raffinose (w/v), 0.1% peptone (w/v), 1.5% NaCl (w/v).

A graph of the mean of the S/N ratios vs. levels (Fig. 5) interprets the best optimized process parameters which were level 3 for pH (pH 11), level 2 for Carbon source (0.5% raffinose w/v), level 2 for NaCl (2.5% NaCl w/v), level 2 for Nitrogen source (0.5% peptone w/v) at 72 h.

The analysis of variance (ANOVA) for the response (the NAG yield) was performed. The results obtained by this Taguchi Orthogonal Array experiment were analyzed by ANOVA and the percentage contributed by each factor was determined. The contribution of pH, Carbon Source (Raffinose), Salt (NaCl concentration), Nitrogen source (Peptone) was 98.31%, 1.12%, 0.03%, 0.53% respectively (Table 4). These results show the contribution among the four factors towards chitin degradation and NAG production is least for the nitrogen source and salt concentration and highest for alkaline pH.

Confirmation of the Taguchi experimental design was carried out by comparing the NAG yield with Taguchi optimum factors and experimental factors (absence of any additional carbon source, nitrogen source and salt with pH 6.8). These results showed it was positively evident that NAG production at 72 h for Taguchi optimum factors (3741 μM) was higher than experimental factors (1649 μM) (Table 5). The unpaired *t*-test (Table 6) for the NAG production has a significant p value of 0.046 (< 0.05).

4. Discussion

Higher yields of desired product need optimization of the process parameters largely involving the use of a good plausible statistical method (Ghaley et al., 2005). According to the available reports Taguchi Design of Experiment is robust design and convincing statisticl method (Roy, 2001). The conventional optimization approach of the process needs measurements and assays followed by analysis steps for the determination of the best parameters. This is limited by the number of experiments, the amount of materials and the time required. Dr. Taguchi (Nippon Telephones and Telegraph company, Japan) has developed a method based on Orthogonal array experiments that provides well balanced experiments and Signal-to-Noise ratios (S/N) that serve as the functions for optimization which help in the prediction of optimum results. The Signal-to-Noise ratios (S/N) can be determined in three different forms such as 1) Smaller the better, 2) Larger the better and 3) Nominal the best.

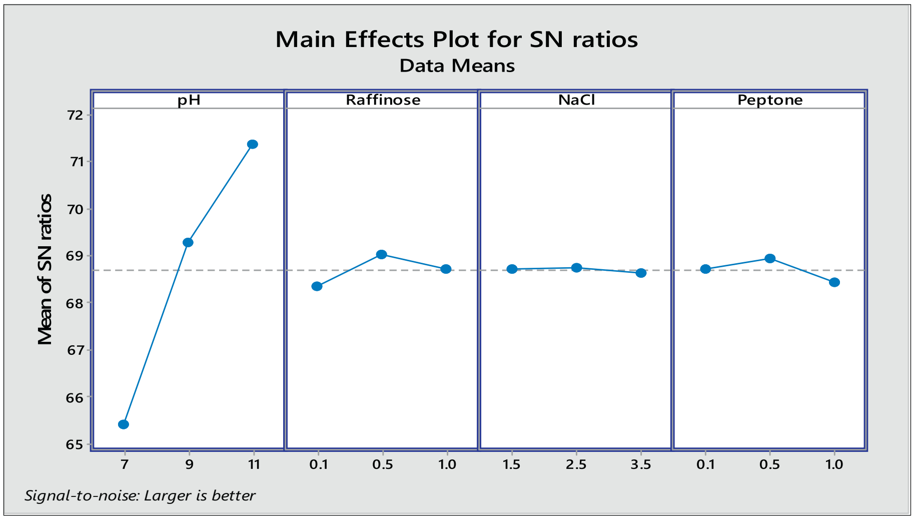

Fig. 5. Mean of S/N ratios were calculated and plotted against four factors (pH, Raffinose, NaCl, Peptone) with their three levels. X-axis shows the factor levels and Y-axis shows the Mean of S/N ratios.

Table 4
Factors and their contribution in production of NAG by RC1830.

Source	Contribution
pH (A)	98.31%
Raffinose (B)	1.12%
NaCl (C)	0.03%
Peptone (D)	0.53%

Table 5
Comparative result of NAG production between optimized and un-optimized conditions showing highest NAG production at every time point (12h – 120h) in optimized conditions.

Time (H)	NAGproduced in micromole (Experimental condition)	NAG produced in micromole (Optimized condition)
12	517	668
24	740	879
36	855	1259
48	1010	1922
60	1245	2355
72	1649	3730
96	1259	2576
120	584	1250

Time: The values are expressed in h.
NAG produced: Represented as micromole.

Table 6
Test of significance result by unpaired *t*-test (performed by GraphPad prism 7 software).

Column B	NAG in micromole (Optimized condition)
vs.	vs.
Column A	NAG in micromole (Unoptimized condition)
Unpaired *t*-test	
P value	0.0462
P value summary	*
Significantly different ($P < .05$)?	Yes

The aim of the current study was to enhance the degradation of the Chitin to obtain N-Acetyl chitooligosaccahride by RC1830. To achieve this the experiment was planned to enhance the production of NAG thereby increasing chitin degradation. Thus, for the S/N ratio the “Larger the better” condition was chosen.

In the present work. Taguchi Orthogonal Array (OA) L_9 $(3)^4$ was used to examine the influence of individual parameters such as pH (A), Carbon Source (B) (Raffinose), NaCl (C), Nitrogen Source (D) (peptone) to increasing the NAG production. This method helped in determining the interactions between various factors and refers to their domination on maximal NAG production with their optimum levels. The result of the statistical analysis demonstrated that maximum contribution of 98.31% was achieved from pH 11. So, pH 11 was the most competent factor for N-Acetyl chitooligosaccahride production by RC1830. From the primary screening process raffinose and peptone was found to be the best Carbon and Nitrogen sources respectively. The ANOVA data showed that Raffinose contributes (1.12%) at 0.5% (w/v) and peptone contributes (0.53%) at 0.5% (w/v), NaCl contributes (0.03) at 2.5% (w/v) and pH at 11 contributes 98.31%.

The proposed production medium for the highest NAG contains 0.5% Raffinose (w/v), 0.5% peptone (w/v), 2.5% NaCl at pH 11. The addition of optimized parameters to the production medium showed a two fold increase in the NAG yield.

The proposed optimized parameters as evident from the data will further be utilized for large scale (Fermentor level) studies for validation and applicability. It can thus be hypothesized that the result will be replicated in the chitin waste degradation studies as well as obtaining higher product yield (NAG and other chitooligosaccharides).

5. Conclusion

This work represents the use of Taguchi experimental design as a very efficient and robust method for determining optimum conditions for chitin degradation demonstrated by N-acetyl chitooligosaccahride production. This process can be applied successfully for the chitin waste degradation process to produce industrially important biomolecules such as chitosan and *N*-acetylchitooligisaccahrides.

The current study is significant by as determining parameters for chitin degradation resulting in eventual valuable product formation that is of prime importance to waste conversion processes.

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References

Aam, B.B., Heggset, E.B., Norberg, A.L., Sørlie, M., Vårum, K.M., Eijsink, V.G., 2010. Production of chitooligosaccharides and their potential applications in medicine. Mar. Drugs 8 (5), 1482–1517. https://doi.org/10.3390/md8051482.

Asadi, Z., Norouzbeigi, R., 2017. Optimization of colloidal nanosilica production from expanded perlite using Taguchi design of experiments. Ceram. Int. 43 (14), 11318–11323. https://doi.org/10.1016/j.ceramint.2017.05.332.

Athreya, S., Venkatesh, Y.D., 2012. Application of Taguchi method for optimization of process parameters in improving the surface roughness of lathe facing operation. Int. Refereed J. Eng. Sci. 1 (3), 13–19.

Aye, K.N., Stevens, W.F., 2004. Improved chitin production by pretreatment of shrimp shells. J. Chem. Technol. Biotechnol. 79 (4), 421–425. https://doi.org/10.1002/jctb.990.

Campana-Filho, S.P., Britto, D.D., Curti, E., Abreu, F.R., Cardoso, M.B., Battisti, M.V., Sim, P.C., Goy, R.C., Signini, R., Lavall, R.L., 2007. Extraction, structures and properties of alpha-AND beta-chitin. Química Nova 30 (3), 644–650. https://doi.org/10.1590/S0100-40422007000300026.

Chen, J.K., Shen, C.R., Liu, C.L., 2010. *N*-acetylglucosamine: production and applications. Mar. Drugs 8 (9), 2493–2516. https://doi.org/10.3390/md8092493.

Dahiya, N., Tewari, R., Hoondal, G.S., 2006. Biotechnological aspects of chitinolytic enzymes: a review. Appl. Microbiol. Biotechnol. 71 (6), 773–782. https://doi.org/10.1007/s00253-005-0183-7.

Elieh-Ali-Komi, D., Hamblin, M.R., 2016. Chitin and chitosan: production and application of versatile biomedical nanomaterials. Int. J. Adv. Res. 4 (3), 411 PMID: 27819009.

Flach, J., Pilet, P.E., Jolles, P., 1992. What's new in chitinase research? Experientia 48 (8), 701–716.

Ghaley, B.B., Hauggaard-Nielsen, H., Høgh-Jensen, H., Jensen, E.S., 2005. Intercropping of wheat and pea as influenced by nitrogen fertilization. Nutr. Cycl. Agroecosyst. 73 (2–3), 201–212. https://doi.org/10.1007/s10705-005-2475-9.

Gross, B.C., Erkal, J.L., Lockwood, S.Y., Chen, C., Spence, D.M., 2014. Evaluation of 3D Printing and Its Potential Impact on Biotechnology and the Chemical Sciences. https://doi.org/10.1021/ac403397r.

Hoell, I.A., Vaaje-Kolstad, G., Eijsink, V.G., 2010. Structure and function of enzymes acting on chitin and chitosan. Biotechnol. Genet. Eng. Rev. 27 (1), 331–366. https://doi.org/10.1080/02648725.2010.10648156.

Hsu, S.C., Lockwood, J.L., 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl. Microbiol. 29, 422–426 PMID: 234719.

Jahanshahi, M., Sanati, M.H., Hajizadeh, S., Babaei, Z., 2008. Gelatin nanoparticle fabrication and optimization of the particle size. Phys. Status Solidi A 205 (12), 2898–2902. https://doi.org/10.1002/pssa.200824329.

Kacker, R.N., Lagergren, E.S., Filliben, J.J., 1991. Taguchi's orthogonal arrays are classical designs of experiments. J. Res. Nat. Inst. Standards Technol. 96 (5), 577. https://doi.org/10.6028/jres.096.034.

Kaushik, G., Thakur, I.S., 2009. Isolation and characterization of distillery spent wash color reducing bacteria and process optimization by Taguchi approach. Int. Biodeterior. Biodegrad. 63 (4), 420–426. https://doi.org/10.1016/j.ibiod.2008.11.007.

Kim, S.K., Rajapakse, N., 2005. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydr. Polym. 62 (4), 357–368. https://doi.org/10.1016/j.carbpol.2005.08.012.

Kirkham, S.G., Samarasinghe, R.K., 2009. Glucosamine. J. Orthopaedic Surg. 17 (1), 72–76. https://doi.org/10.1177/230949900901700116.

Minitab, I., 2014. MINITAB Release 17: Statistical Software for Windows. Minitab Inc, USA.

Mishra, M., Thakur, I.S., 2010. Isolation and characterization of alkalotolerant bacteria and optimization of process parameters for decolorization and detoxification of pulp and paper mill effluent by Taguchi approach. Biodegradation 21 (6), 967–978. https://doi.org/10.1007/s10532-010-9356-x.

Mnif, I., Sahnoun, R., Ellouze-Chaabouni, S., Ghribi, D., 2014. Evaluation of *B. subtilis* SPB1 biosurfactants' potency for diesel-contaminated soil washing: optimization of oil desorption using Taguchi design. Environ. Sci. Pollut. Res. 21 (2), 851–861. https://doi.org/10.1007/s11356-013-1894-4.

Oskouie, S.F.G., Tabandeh, F., Yakhchali, B., Eftekhar, F., 2007. Enhancement of alkaline protease production by *Bacillus clausii* using Taguchi experimental design. Afr. J. Biotechnol. 6 (22). https://doi.org/10.5897/AJB2007.000-2408.

Ray, L., Suar, M., Pattnaik, A.K., Raina, V., 2013. Streptomyces chilikensis sp. nov. a halophilic streptomycete isolated from brackish water sediment. Int. J. Syst. Evol. Microbiol. 63 (8), 2757–2764. https://doi.org/10.1099/ijs.0.046284-0.

Reissig, J.L., Strominger, J.L., Leloir, L.F., 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. J. Biol. Chem. 217 (2), 959–966 PMID:13271455.

Roy, R.K., 2001. Design of Experiments Using the Taguchi Approach: 16 Steps to Product and Process Improvement. John Wiley & Sons.

Shahidi, F., Abuzaytoun, R., 2005. Chitin, chitosan, and co-products: chemistry, production, applications, and health effects. Adv. Food Nutr. Res. 49, 93–137. https://doi.org/10.1016/S1043-4526(05)49003-8.

Souza, C.P., Almeida, B.C., Colwell, R.R., Rivera, I.N., 2011. The importance of chitin in the marine environment. Mar. Biotechnol. 13 (5), 823. https://doi.org/10.1007/s10126-011-9388-1.

Suresh, P.V., 2012. Biodegradation of shrimp processing bio-waste and concomitant production of chitinase enzyme and *N*-acetyl-D-glucosamine by marine bacteria: production and process optimization. World J. Microbiol. Biotechnol. 28 (10), 2945–2962. https://doi.org/10.1007/s11274-012-1106-2.

Vaidya, R., Vyas, P., Chhatpar, H.S., 2003. Statistical optimization of medium components for the production of chitinase by Alcaligenes xylosoxydans. Enzym. Microb. Technol. 33 (1), 92–96. https://doi.org/10.1016/S0141-0229(03)00100-5.

Vijayaraj, S., Varatharajulu, M., 2015. Optimisation of process parameters of wet scrubbing of biogas from night soil and vegetable waste using historical and Taguchi design. Progr. Indus. Ecol. Int. J. 9 (2), 168–181. https://doi.org/10.1504/PIE.2015.073082.