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Optimization Studies of Alkaline Cellulase from *Bacillus licheniformis* SM1 Isolated from Rice Agricultural Soil using Taguchi Methodology

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The aim of the present work was to screen microbes related to alkaline cellulase production from rhizosphere of rice field soil from Phagwara, Punjab. Out of 9 isolates screened, isolate 6th designated as SM1 was selected for alkaline cellulase study. Results of 16S rRNA revealed this strain as *Bacillus licheniformis*. Various medium conditions affecting cellulase activity such as pH, C/N ratio, Tween-20 and lactose were optimized using L9 conditions by Taguchi methodology in submerged conditions. Factors having significant impact over alkaline cellulase activity were in order pH > C/N ratio > Tween-20 > Lactose. The most optimum conditions for cellulase production was pH 9; C/N, 1:2; Lactose, 1 % (w/v) and Tween 20, 1 %(v/v) at 50 ^{0}C.

Key words: Optimization, Alkaline Cellulase, activity, CMCase,

Alkaline cellulases are known to operate under high pH. Various fungi secrete cellulases such as *Aspergillus, Fusarium, Humicola, Melanocarpus, Penicillium Trichoderma,* but none is stable and active in alkaline pH. Some thermophiles and extremophiles secrete cellulases which are stable and works in the alkaline range pH (9-12) e.g.. *Myceliophthora thermophila* produces alkaline cellulases (pH 4-12); Bacillus sp KSM 635 has been patented for active cellulase in the pH range (4-11)[1]. A thermophillic microbe, *Aneurinibacillus thermoaerophilus WBS2 was* isolated by Acharya and Chaudhary (2012) which produced alkaline cellulase active at pH 9.0 and temperature 65°C[2]. A halophilic alkaline endoglucanase was purified from *Bacillus licheniformis* isolated from soils of Lake Van Soda in Turkey. The reported optimum pH and temperature were 10.0 and 30°C, respectively In addition, the enzyme stability was reported upto100°C and in 6 hrs. in 7 to10 % of NaCl [3]. *Bacillus cereus* MRK1was used for production of alkaline cellulase in optimized conditions for its specific applications in the Bio-stoning activity. The reported optimized condition was pH 8.0, temp 32 °C and enzyme activity was reported up to 102 U/ml[4]. Another thermo-stable alkaline cellulase was reported from Bacillus sp KSM S237, which produced enzyme in the pH range 8.6-9.0 and at temp. 45 ^{o}C. In addition, the enzyme was able to hydrolyze CMC, lichenan cellotriose derivatives and Cellotetraose[6,7]. Owing to increased applications of alkaline cellulase in Bio-stoning and laundry detergents, isolation of alkaline cellulases was done from bacterium isolated from rhizospheric plane of the soil and optimization of factors

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responsible for inducing cellulase production was done using Taguchi Methodology.

MATERIALS AND METHODS

Isolation and screening of microbes

Soil samples were collected at a depth of 10cm from the rhizospheric plane of rice field, Phagwara, in a sterilized bottle**.** The soil samples were diluted up to 10^{-6} and plated on 2% CMC agar media and incubated at 37° C at varying pH range (8-10) for 7 days. The plates were flooded with 0.1 % Congo red dye and washed with 1M NaCl. The cellulase producing colonies were screened on the basis of the zone of hydrolysis obtained. The bacterial isolates with prominent zone was selected and was further used in the study.

Bacterial Identification

The screened isolate was characterized by biochemical tests and the parameters investigated were Indole production, Methyl red, VP reaction, Citrate utilization, Urease, Catalase, Litmus milk, H_2S production, TSI, Dextrose fermentation, Lactose fermentation, Sucrose fermentation, Mannitol fermentation and the species was confirmed by 16S rRNA analysis.

Enzyme Production

The enzyme production was done in following media (g/L) Peptone-1g, K_2HPO_4-0.5g, $MgSO_4$-0.5g, CMC-10g, Na_2CO_3-10g. The media was autoclaved and 5ml of overnight grown bacterial culture was inoculated and was kept in a shaker incubator at 37 ^{0}C for 48 hours. After 48 hours, the culture was centrifuged for 30 mins at 5000 g and the supernatant was used for further analysis.

Enzyme Assay

The crude enzyme obtained after ammonium sulfate precipitation was used for Cellulase assay using DNS method[8]. Cellulase activity was measured by Ghose Method[8]. 0.5 ml of substrate and crude enzyme were mixed and incubated at 50° C for 30 min. The enzyme – substrate reaction was terminated by adding 3 ml DNS reagent and was boiled vigorously at 100o C for 5 minutes. It was then allowed to cool down and then 20 ml of distilled water was added to each mixture. This solution was mixed properly by inverting the test tube and the OD was taken at 540 nm.

Optimization of factors

Optimization of factors was done with Taguchi methodology using the Qualitek-4 software. Various steps in optimization methodology were

a) Factors identification and determination of levels

b) Proper selection of orthogonal arrays

c) Experimental setup to get the response

d) Factors were determined that influence the production of enzymes.

e) Then optimum conditions were predicted, analyzed and validated by ANOVA.

Choice of factors

For alkaline cellulase production, design of experiments was done using factors pH, C/N, Lactose and Tween 20 at three different conc. (levels) as shown in Table 1. L_9 design was obtained in Qualitek 4 software and the experiment was performed according to the Orthogonal Array (OA) L_9 design.

Analysis of Experiment

The obtained experimental data were processed in the Qualitek -4 software with bigger is better quality characteristics for the determination of the optimum conditions for cellulase production and to identify the individual factors influencing cellulase production.

In Taguchi's method, quality is measured by the deviation of a characteristic from its target value and loss function [L (y)] is developed for the deviation as represented by $L(y) = kx(y-m)^2$, where k denotes the proportionality constant, m represents the target value, and y is the experimental value obtained after each run [20]. In case of bigger is better quality characteristics, the loss function can be written as L (y) = k x (1/y), and expected loss function can be represented by

$$E[L(y)] = k \times E(1/y^2) \quad ....(1)$$

Wheree E $(1/y^{j})$ can be estimated from a sample of n as

$$\sum_{i=1}^{n}(1/Yi^2)/n \sum_{i=1}^{n}(1/Yi^2)/n \quad ...(2)$$

The results obtained after the data processing by the Qualitek-4 software are shown in Tables 4-8

Validation

To validate the methodology, optimized conditions were used to get the results.

Software

Qualitek-4 software (Nutek Inc., Michigan, USA; Roy, 2001) for automatic design of experiments using the Taguchi approach was used in the present study. Qualitek-4 software is equipped to use L-4 to L-64 array along with the selection of 2–63 factors with 2–3 and 4 levels to each factor.

RESULTS AND DISCUSSION

Results of screening have been shown in Table 2. We can see from the table that, out of nine isolates screened at varying pH 8-10, only 6th isolate at pH 9 showed very good growth. In addition the isolate was having the maximum zone of hydrolysis. Biochemical identification based on Bergeys manual shows positive test for sucrose fermentation and litmus milk test giving indication of Bacillus.16S rRNA phylogenetic analysis showed, it is 99% similar to *Bacillus licheniformis* and was named as *Bacillus licheniformis* SM1. This strain has been submitted to GenBank with accession number KF522027.1

B. licheniformis, is reported to produce multiple enzymes such as tannase, alkaline protease, keratinase, thermo stable alpha amylase, chitinase and various other enzymes such as pectate, lyases, lipases, along with various polysaccharides degrading enzyme[9-14] and because of these various species of bacillus have been patented due to its applications in Industrial use[1]. A variety of source have been used from where alkaline cellulase secreting microbes have been isolated such as soil[15] , from rhizospheric soil[13] from marine sediment[16]. Veith *et al* (2004) reported complete sequence of *Bacillus licheniformis* DSM[13], and revealed some interesting finding that it possess many enzymes such as glyoxylate bypass, and glyoxylate reductase which reveals that it has the capability to grow anywhere in any condition.It also has good ability to grow on acetate and 2,3-butane diol[14].

Optimization of factors

According to literature survey, some of the prominent nutritional media components such as pH, Cellulose, Peptone, Tween 20 and lactose has an important role in inducing alkaline cellulase production. For this, nine trials were obtained for four factors at three levels, and designated as L_9 design as shown in Table.4

In addition, experiments were performed according to these trials and responses obtained in triplicate were entered and the software automatically calculates S/N ratio as shown in Table 4. Based on the S/N ratio, individual important factors, effecting enzyme production can be estimated based on highest positive or negative value.

Average effect of factors

The results of average effect of Individual factors such as pH, C/N ratio lactose and tween 20 have been shown in Figure 3.

Table 1. Factors and their assigned levels

S. No.	Factors	Units	Level 1	Level 2	Level 3
1	pH		8	9	10
2	C/N Ratio	(w/v)	1:1	2:1	1:2
3	Lactose %	(w/v)	0.5	1.0	1.5
4	Tween-20 %	(v/v)	1	2	3

Table 2. Screening conditions

Conditions#	pH	Growth	Activity (Zone of Hydrolysis)
1	8	+ve	+ve
2	8	++ve	+ve
3	8	-ve	-ve
4	9	+ve	+ve
5	9	-ve	-ve
6	9	++ve	++ve
7	10	+ve	+ve
8	10	+ve	+ve
9	10	+ve	+ve
Control	8	+ve	+ve
Control	9	+ve	+ve
Control	10	+ve	+ve

Note: -ve No growth; +ve growth ; ++ very good growth

Results of impact of pH over alkaline cellulase activity has been shown in Fig 3a which revealed that with increase in pH, enzyme activity increases from 8 to 9 then decreases from 9 to 10. Therefore enzyme works best at pH 9. Similarly Aygan *et al.* (2008) isolated a bacterial strain *Bacillus sp C14 which* was showing maximum enzyme activity at pH 9 [3]. Three different strains of *Bacillus viz.Bacillus sp*. KSM-19, KSM-64, KSM-520 were isolated which were showing optimal enzyme production in the range of 8.5 to 9.5 while *Bacillus circulans* KSM N257 produced

Table 3. Biochemical Identification

Biochemical tests	Observation	Results
Indole production.	No Colour change	-ve
Methyl red	No Colour change	-ve
Voges Proskauer	no ring formation	-ve
Citrate utilization	no Colour change	-ve
Urease	No Colour change	-ve
Catalase	No bubble formation	-ve
Litmus milk	White Colour with purple band on the top	+ve
H2S production	No blackening of the media	-ve
TSI	No change	-ve
Dextrose fermentation	No Colour change	-ve
Lactose fermentation	No Colour change	-ve
Sucrose fermentation	Colour changes from Red to yellow	+ve
Mannitol fermentation	No Colour change	-ve

Table 4. Trial conditions as per the Orthogonal Array L_9 Designs/N ratio calculation based on the response entered into result column. Based on the S/N ratio, individual important factor can be calculated

Trial conditions	pH	Lactose (%)	C/N (%)	Tween 20(%)	Enzyme activity 1(IU)	Enzyme activity2 (IU)	Enzyme activity3 (IU)	S/N Ratio
1	8	0.5	1:1	1	0.188	0.197	0.180	-14.52
2	8	1	2:1	2	0.254	0.226	0.257	-12.238
3	8	1.5	1:2	3	0.234	0.240	0.223	-12.69
4	9	0.5	2:1	3	0.186	0.191	0.177	-14.686
5	9	1	1:2	1	0.306	0.291‘	0.317	-10.34
6	9	1.5	1:1	2	0.380	0.400	0.320	-8.835
7	10	0.5	1:2	2	0.043	0.041	0.048	-27.188
8	10	1	1:1	3	0.097	0.103	0.088	-20.41
9	10	1.5	2:1	1	0.154	0.166	0.174	-15.701

Table 5. Interaction between factors and their Severity Index (SI) above > 30 % shows significant impact over the activity

#	Interacting factor Pairs (Order based on SI)	Columns	SI (%)	Col.	Opt.
1	C/N x Tween %	2*4	29.34	6	[3,2]
2	Lactose % x C/N	2*3	22.71	1	[3,1]
3	pH x Lactose %	1*3	22.15	2	[2,1]
4	Lactose % x Tween %	3*4	6.05	7	[1,2]
5	pH x C/N	1*2	5.62	3	[2,3]
6	pH x Tween %	1*4	2.11	5	[2,2]

endoglucanase at pH 8.5 and *Bacillus licheniformis* C108 produced endoglucanases at pH 10 [6-9,17].

The results of C/N ratio have been exhibited in Figure 3b. From the result, it can be concluded that C/N ratio 1:2 has a positive impact over alkaline cellulase production. By increasing the C/N ratio, a continuous increase in enzyme activity was noted. Effect of nitrogen supply has also been examined by Gomaa et al 2012 which reveals that enzyme production and activity increases on high nitrogen supply[13]. The effect of lactose addition has been evidenced in Figure 3 c which exhibits that the addition of lactose from 0.5-1% has a positive effect over enzyme activity (from level 1 to 2) after that, activity decreased slowly. Thus, it can be concluded that, 1% lactose is acting as an inducer in enhancing production of cellulase. The average effect of Lactose as inducer was studied by Douglas, 2001 who concluded that activation of the Lac operon induces beta galactosidase operon as a result cellulase production increases[18]. Hmad *et al* (2014) optimized and studied the alkaline cellulase production from *Stachybotrys* strain. He further reported that glucose and lactose repressed the CMCase production, but induces beta -glucosidase. Some substrate like CMC, Avicel, and wheat bran was reported to be a good activator of enzyme CMCase. In addition, pH was also an important factor in alkaline endoglucanase secretion[19]. The effect of surfactant over cellulase production has been shown in Fig 3d. It can be observed that Tween 20 is very effective at level 1 further it has decreased impact over cellulase production. Since, surfactant is involved in media transport and save the cells from any stress condition.

Table 6. Main effects determination based on difference of change in level

Column # factors	Level 1	Level 2	Level 3	L3-L1
1. pH	-13.149	-11.28	-21.1	-7.952
2. C/N	-18.798	-14.32	-12.409	6.388
3. Lactose	-14.588	-14.20	-16.739	-2.152
4.Tween20%	-13.52	-16.08	-15.929	-2.41

Table 7. ANOVA (analysis of Variance) analysis

Col# factor	DOF (f)	Sum of sqrs(S)	Variance(V)	Pure sum (S')	Percent p (%)
1. pH	2	162.964	81.482	162.964	64.92
2. C/N	2	64.466	32.233	64.466	25.681
3. Lactose %	2	11.177	5.588	11.177	4.452
4. Tween 20	2	12.412	6.206	12.412	4.944
Other error	0				
Total	8	251.021			100.00 %

Table 8. Optimum Conditions and Performance obtained after analysis of variance which yields optimum factors along with their level and concentration

ColumnFactor	Level description	Level	Contribution
1. pH	9	2	3.891
2. C/N	1:2	3	2.769
3. Lactose %	1	2	0.970
4. Tween 20	1	1	1.658
Total contribution from all factors			9.288
Current Grand Average of performance			15.179
Expected result at optimum conditions			5.891

The interaction between two factors gives a better insight into the overall process analysis. Any individual factor may interact with any or all others factors creating the possibility of the presence of a large number of interactions. This kind of interaction is possible in Taguchi DOE methodology. Interactions under study help to know influence of two individual factors at various levels of the interactions. In Table 5, the column represents the locations to which the interacting factors were assigned. Interaction SI presents 100 % of SI for a 90-degree angle between the lines while 0 % SI is for parallel lines. Reversed column should be reserved if this interaction effect has to be studied. "Levels" indicate the factor levels desirable for the optimum condition (based on the

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Table 7. Interaction between factors and their Severity Index (SI) above > 30 % shows significant impact over the activity

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other error	0				
Total	8	251.021			100.00 %

Table. 9. Optimum Conditions and Performance obtained after analysis of variance which yields optimum factors along with their level and concentration. Based on this total contribution from all factors were 9.288 while current grand average of performance was 15.179. Therefore expected result at optimum condition was 5.891 units.

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first two levels).

Interaction show that very few factors are interacting significantly. Since most of the factors show SI % below 29, still from the data it can be concluded that Tween 20 has a significant impact with other factors such as C/N ratio and Lactose which was according to expectation.

Explanations of Columns of Table

Columns- Represent the column locations to which the interacting factors are assigned

SI- Interaction Severity Index (100 % for 90 degrees angle between the lines, 0 % for parallel lines)

Col- Shows column that should be reversed if this interaction effect were to be studied (2-L factors only)

Opt- Indicates factors levels desirable for the optimum condition (based strictly on the first 2 levels).

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 condition.

Main effects determination

The factors showing significant influence on cellulase production have been shown in Table. 6 based on difference in the level. The highest negative and positive value shows the greatest

Fig. 1. Phylogenetic analysis according to 16S rRNA analysis. Gene has been submitted to GenBank: KF522027.1

impact. Therefore it can be concluded that pH and Carbon /Nitrogen ratio are the most significant factors which is confirmed from Fig. 4 Bar diagram and pie chart.

Analysis of Variance (ANOVA)

ANOVA analysis as shown in Table 7 determines overall percent contribution of factors over enzyme activity. Here pH has 64.92 % contribution, C/N 25.681 %, Lactose 4.452 % and Tween-20 has 4.944 % contribution. This fact can be verified by the pie chart and Bar diagram as shown in Fig. 4. These two graphs and ANOVA

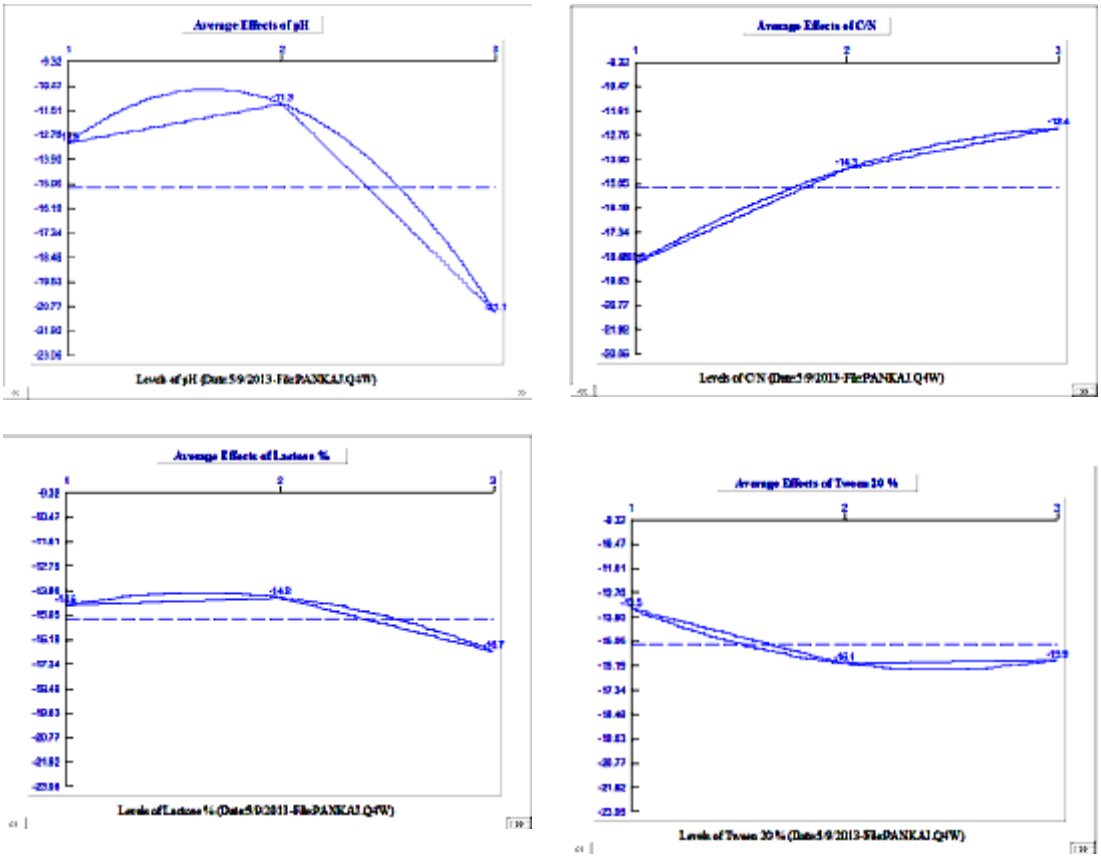

Fig. 3. Average effects of individual factors (a) pH (b) C/N (c) Lactose as inducer of enzyme production (d) surfactant Tween 20, over alkaline cellulase production

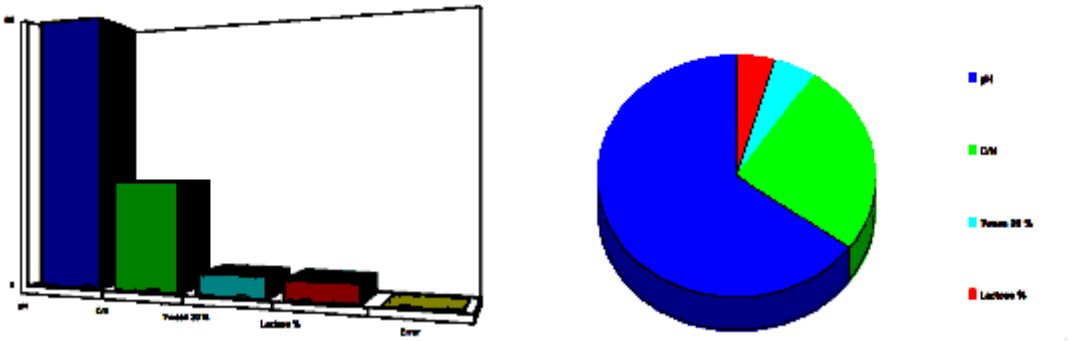

Fig. 4. Factors and their contribution presented in A. Bar Diagram B. Pie Chart

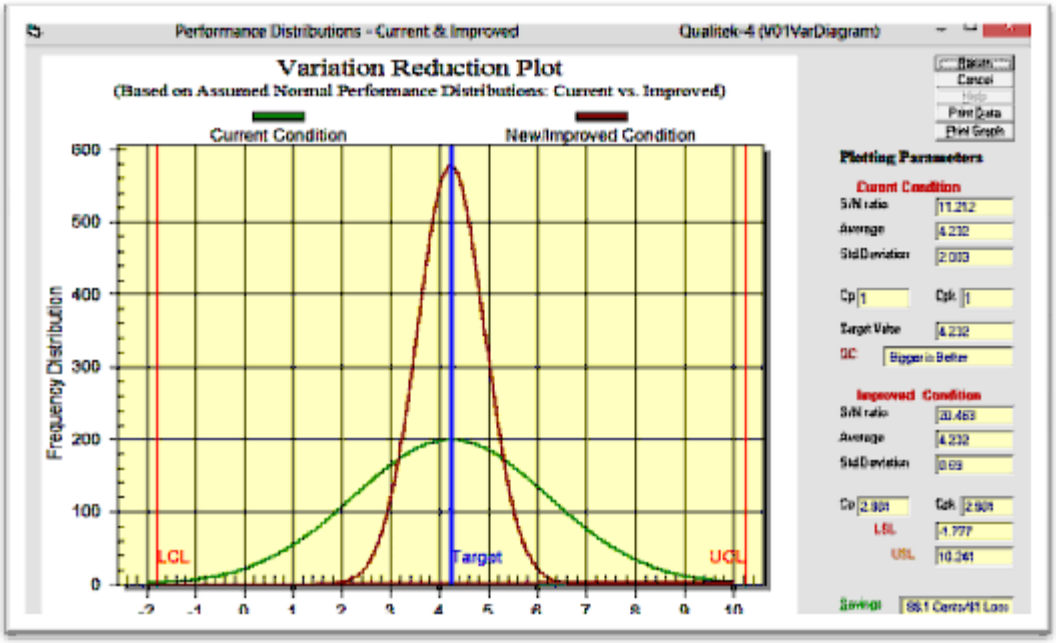

Fig. 5. Performance distributions- Current and improved

analysis showed that pH has major contribution (64.92 %) to the cellulase production. The 2nd major contribution was of C/N % for enzyme activity. It contribution was 25.681 % to the enzyme production. Lactose and Tween 20 contributed 4.452 % and 4.944 % respectively.

After complete analysis of each factor, optimization of these factors was done by this software. Once we get the optimum levels at which enzyme activity is highest, we can expect that these set of conditions will produce maximum cellulases. From **Table. 8**, it is clear that maximum enzyme will be produced at pH of level 2 i.e. pH 9, C/N of level 3 i.e. 1:2 %, Lactose of level 2 i.e. 1 % and Tween-20 of level 1 i.e. 1 %. Total contribution from all these factors was estimated to be 9.288 and current grand average of performance is 15.179. At these optimum conditions, Enzyme activity that can be expected is 5.891 units. Further, these conditions can be validated performing an experiment with these set of optimum conditions.

CONCLUSION

A novel Alkaline Cellulase bacteria was successfully isolated and identified as *Bacillus licheniformis* from rice field soil, which is able to grow maximum at pH 9. Further, to enhance enzyme production, media factors such as pH, C-source, N-source, and Tween 20and Lactose were optimized. Significant effect of each factor was as follows pH > C/N > Tween-20 > Lactose over alkaline cellulase production. The most optimum condition which was obtained for maximum production of Cellulase was pH 9; C/N, 1:2; Lactose, 1 % (w/v) and; Tween 20, 1 %(v/v). Based on this total contribution from all factors were 9.288 while current grand average of performance was 15.179.

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REFERENCES

1. Ito, Susumu. Alkaline cellulases from alkaliphilic *Bacillus*: enzymatic properties, genetics, and application to detergents. *Extremophiles* ,1997; **1**(2): 61-66.
2. Acharya, Somen, and Anita Chaudhary. Alkaline cellulase produced by a newly isolated thermophilic Aneurini*Bacillus* thermoaerophilus WBS2 from hot spring, India. *African J.Microbiol. Res.*, 2012; **6**(26): 5453-5458.

3. Aygan, A. S. H. A. B. I. L., and B. U. R. H. A. N. Arikan. A new halo-alkaliphilic, thermostable endoglucanase from moderately halophilic *Bacillus* sp. C14 isolated from Van soda lake. *Int J Agri Biol.,2008;***10**: 369-374.
4. Kumar, D.J. Mukesh, *et al*. Optimization of *Bacillus* cereus MRK1 cellulase production and its Biostoning activity. *Der Pharmacia Lettre.,* 2012;**4**: 881-888.
5. Raddadi, Noura, *et al*. The most important *Bacillus* species in Biotech.*Bacillus thuringiensis Biotech.* Springer Netherlands., 2012: 329-345.
6. Hakamada, Yoshihiro, *et al*. Thermostable alkaline cellulase from an alkaliphilic isolate, *Bacillus sp.* KSM-S237. *Extremophiles.,* 1997; **1**(3): 151-156.
7. Shikata, Shitsuw, *et al*. Alkaline Cellulases for Laundry Detergents: Production by Alkalophilic Strains of *Bacillus* and Some Properties of the Crude Enzymes Microbiol. & Fermentation Industry. *Agri Biol. Chem.,*1990; **54**(1): 91-96.
8. Ghose TK Measurement of cellulase activity, *Pure and Appl. Chem.,* 1987; **59**: 257-268.
9. Das Mohapatra, P. K., *et al*. Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology.*Food Res.I.,* 2009;**42**(4): 430-435.
10. Sellami-Kamoun, Alya, *et al*. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. *Microbiological Res.,* 2008*;* **163**(3): 299-306.
11. Lin, Xiang, *et al*. Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Applied and Enviro. Microbiol.,*1992; **58**.(10): 3271-3275.
12. Niu, Dandan, *et al*. High yield recombinant thermostable α-amylase production using an improved *Bacillus licheniformis* system. *Microbial cell factories*.,2009; **8**(1):58.
13. Gomaa, Eman Zakaria. Chitinase production by *Bacillus* thuringiensis and *Bacillus licheniformis*: their potential in antifungal biocontrol. *The J. of Microbiol.,*2012; **50**(1): 103-111.
14. Veith, Birgit, *et al*. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great Ind. potential. *J. of Mol. Microbiol. and Biotech.,*2004; **7**(4): 204-211.
15. Singh, Jagtar, Navneet Batra, and Ranbir Chander Sobti. Purification and characterisation of alkaline cellulase produced by a novel isolate, *Bacillus sphaericus* JS1. *J. of Ind. Microbiol. and Biotech.,*2004; **31**(2):51-56.
16. Smitha, S., and S. G. Bhat. Thermostable Bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. *J. of Applied Microbiol.,* 2013; **114**(3): 688-694.
17. Aygan, Ashabil, Lutfiye Karcioglu, and Burhan Arikan. Alkaline thermostable and halophilic endoglucanase from *Bacillus licheniformis* C108. *African J. of Biotech.,*2011*;* **10**(5): 789-796.
18. Juers, Douglas H., *et al*. A structural view of the action of *Escherichia coli* lac Z B-galactosidase. *Biochemistry*., 2001; **40**.49 : 14781-14794.
19. Hmad, I. B., Abdeljalil, S., Saibi, W., Amouri, B., & Gargouri, A. Medium Initial pH and Carbon Source Stimulate Differential Alkaline Cellulase Time Course Production in *Stachybotrys microspora. Applied Biochem. and Biotech*., 2014; **172**(5) :2640-2649.
20. Bhatt, S. M., & Srivastava, S. K. Lactic acid production from cane molasses by *Lactobacillus delbrueckii* NCIM 2025 in submerged condition: optimization of medium component by Taguchi DOE methodology. *Food Biotechnology*., 2008; **22(**2): 115-139.