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PROTONATED CRAB SHELL WASTE AS FUNGAL INHIBITOR

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INTRODUCTION:

In the present time, it becomes very unlikely to overlook fungal contamination, which is a serious issue in most tropical countries. India, one of the largest tropical regions, is suffering from fungal contamination in various sectors which may damage the development of the whole nation. For counteracting this problem, there is an intensive rise in the usage of chemicals to inhibit the growth of various fungi due to their compatibility. However, these chemicals are composed of harmful and toxic compounds that may not only eliminate fungi but also risk human health. India is also a country which is rich in marine life and abundant in sea resources. Utilization of these wastes from sea resources may potentially be used to resolve the issue of fungal contamination. This also addresses the issue of managing this waste in a way which can be beneficial for society. Therefore, the aim of this research was to analyze the usage of protonated crab shell waste as an alternative to inhibit the contamination and growth of Aspergillus niger . The analysis involved statistically studying the appropriate concentration of chitosan(extracted from crab shells) in the optimum concentration of acetic acid and glucose in growth media to bring about maximum inhibition. Further, the structure of chitosan was studied using mass spectroscopy and its interaction with the optimum concentration of acetic acid was analyzed.

Materials and Method

[1] Chitosan was extracted directly from crab shell waste. The procedures involved in the extraction of chitosan were deproteination, demineralization, and decolorization.[2] Agar diffusion test was performed multiple times to determine the best dose on inhibiting the growth of Aspergillus niger . For performing the agar diffusion test, each petri dish composed of isolated Aspergillus niger and sufficient amount of agar. To observe the inhibition zone, Petri dishes were incubated for 24 hours.[3] The first analysis was performed to analyze the inhibition of fungal growth shown by varying concentration of acetic acid in the presence and absence of 1 g chitosan in the varying amount of glucose in the culture media. Samples consisting of 1 g of chitosan in varying concentration of acetic acid were tested in 0%, 0.25%, 0.50%, 0.75% and 1% glucose in culture media. At the same time, samples consisting of pure acetic acid in different concentrations were also tested in the above-mentioned concentrations of glucose in culture media and the diameter of inhibition zones(measured by using a vernier caliper) observed were noted for each sample.[4] The ideal concentration of acetic acid and glucose in culture media identified in the first analysis was then used for determining the most effective

chitosan concentration in inhibiting the growth of Aspergillus niger. The different concentrations of chitosan studied were 2%, 1%, 0.5%, 0.25% and 0.125% (w/v). At the same time, in protonated chitosan solutions with the above-mentioned concentrations were also tested for comparison. The diameter of the inhibition zones observed in each test was measured using vernier caliper.[5] Each agar diffusion test was conducted repeatedly. Results obtained were analyzed and verified using statistical analysis. [6] Mass spectroscopy, spectrophotometer and attention weight analysis were used to study the reactions involved and the final structure responsible for fungal inhibition.

Observation

Table 1 shows the zone of inhibition observed for the varying concentration of acetic acid in the presence and absence of 1 g chitosan in different concentrations of glucose in culture media.

Table 1:

Variation in acetic acid with 1g chitosan	Zone of inhibition(cm) for different % of glucose in media					Avg
	0%	0.25%	0.50%	0.75%	1%	
85%	4.95	5.1	4.9	5.05	4.8	4.96
75%	4.25	4.35	4.2	4.3	4.27	4.274
50%	2.8	2.66	3	2.76	2.7	2.784
25%	2	1.95	2.05	2.05	1.95	2
1%	0	0	0	0	0	0
Variation in acetic acid						
85	3.95	3.9	4.05	3.82	3.97	3.938
75	2.35	3.17	3.27	3.15	3.22	3.032
50	1.8	1.75	1.58	1.95	1.71	1.758
25	1	0.95	0.9	1	1	0.97
1	0	0	0	0	0	0

Analyzing the average zone of inhibition in the presence and absence of 1 g of chitosan (Figure 1) it was evident that the presence of chitosan maximized the zone of inhibition thereby acting as an inhibitor. Since the presence of chitosan was effective in maximizing inhibition it was further analyzed. Figure 2 shows the inhibition exhibited by varying concentrations of acetic acid with 1 g of chitosan in different concentration of glucose in media. From the figure, it can be seen that 85% concentration of acetic acid is ideal as it exhibits a maximum zone of inhibition. Further, to identify the ideal concentration of glucose in media, the results obtained at each concentration of acetic acid were compared with the different concentration of glucose present in media(Figure 3). Figure 3 clearly shows maximum inhibition was seen in 0.25% concentration of glucose in media with 85% acetic acid concentration in the presence of 1g chitosan.

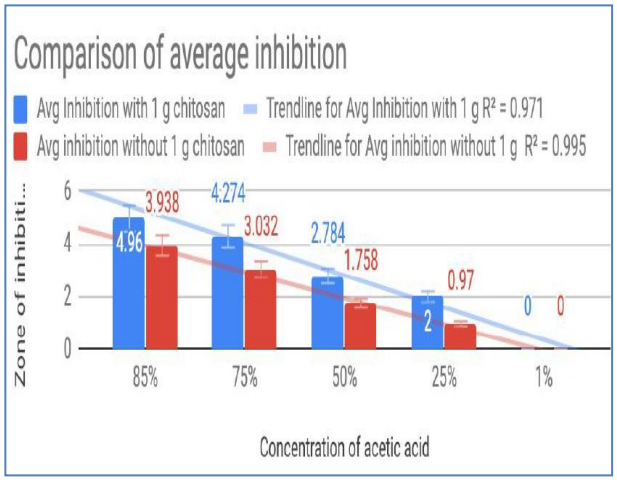
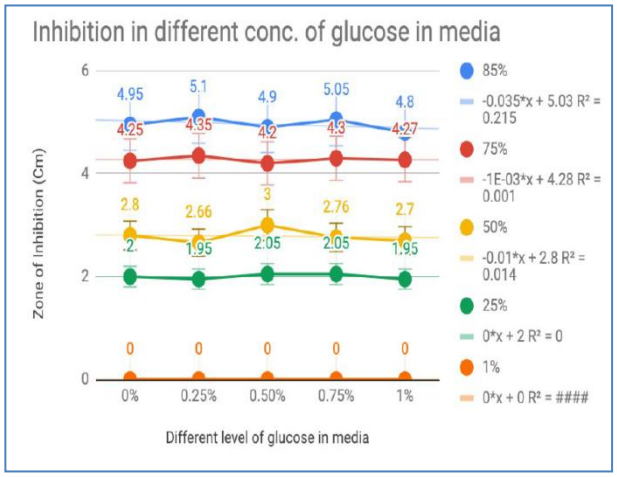


Figure 1:



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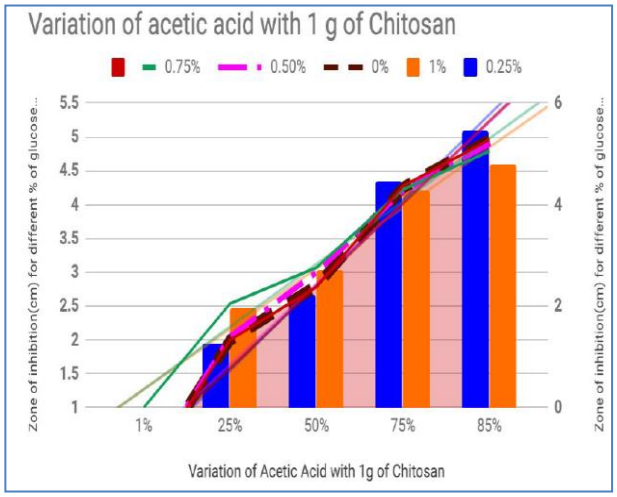


Figure 3:

Table 2:

%chitosin in 85%acetic acid (0.25% glucose media)	Zone of inhibition(cm) observed on different days						Avg
	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	
2%	4.25	3.51	4.09	4.54	3.8	4.38	4.095
1.50%	5	4.26	4.84	5.63	4.89	5.47	5.015
1%	5.25	4.51	4.96	5.41	5.86	6.31	5.383333333
0.75%	5.6	4.77	5.35	6.14	6.72	7.3	5.98
0.50%	5.99	5.25	5.83	6.62	7.2	7.78	6.445
0.25%	5.7	4.87	5.32	5.77	4.94	5.39	5.331666667
0.13%	4	3.17	3.75	4.2	3.37	3.95	3.74

Table 2 Therefore, it became evident after the first analysis that 85% acetic acid with 1 g of chitosan in 0.25% concentration of glucose in culture media was ideal and exhibited maximum inhibition. Considering this result the second analysis was performed with 855 acetic acid and 0.25% glucose in culture media tested with different concentrations of chitosan. Table 2 shows the zone of inhibition(cm) observed for different concentrations of chitosan on Day 10, Day 11 uptill Day 15. Figure 4 shows the comparison of average zone of inhibition observed at different concentrations of chitosan. Maximum average inhibition was observed at 0.50% concentration. Further, comparative analysis as in Figure 5 shows that the individual contribution in inhibition of fungal growth was maximum for 0.50% concentration of chitosan. When the inhibition at each concentration of chitosan was compared day-wise(Figure 6) it was observed that the highest total inhibition by all concentrations was seen on Day 15 and the individual inhibition shown by 0.50% chitosan was also highest on Day 15 thereby pointing out to the fact that 0.50% concentration of chitosan was most effective and exhibited maximum inhibition.

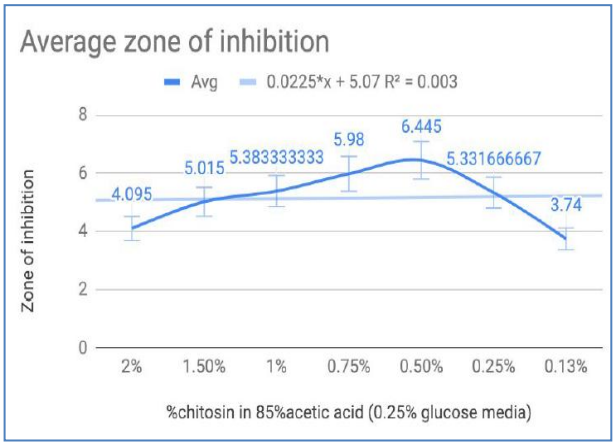
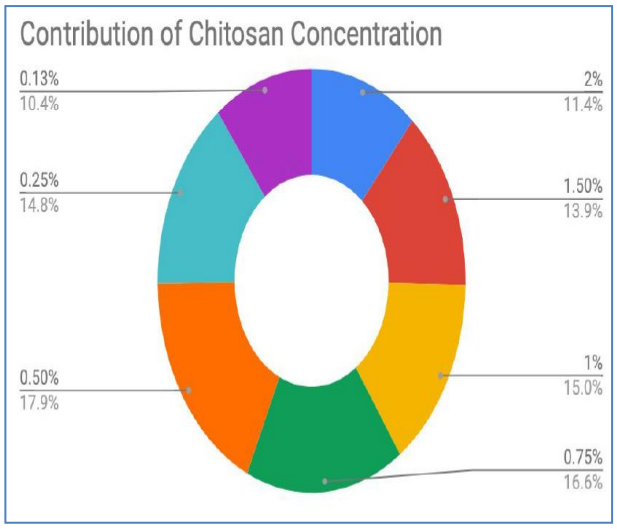


Figure 4:



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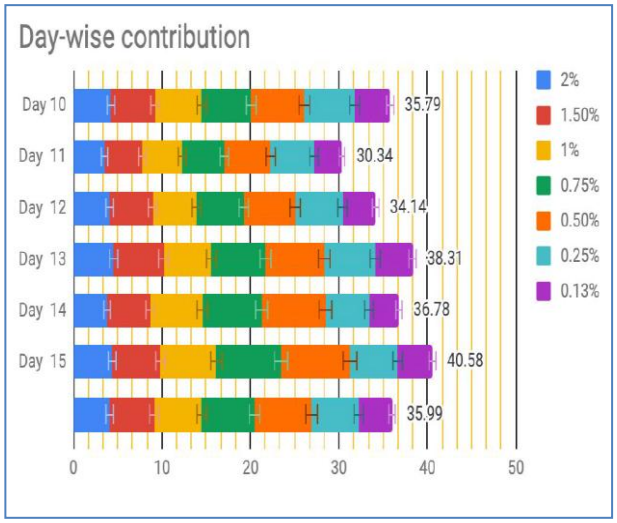


Figure 6:

The difference in the result obtained at different concentrations of chitosan was analyzed statistically using analysis of variance(ANOVA). The factor of % of chitosan present was studied for response in terms of inhibition observed at 5% level of significance.The p-value obtained for the process(<0.05) showed that the hypothesis of no difference could be rejected. Therefore, the difference was statistically significant pointing out to the fact that the observation of maximum inhibition at 0.50% chitosan was not a chance event. Table 3 shows the analysis and Table 4 shows the mean, standard deviation and 95% CI for each concentration of chitosan. The obtained result has been plotted in Figure 7.

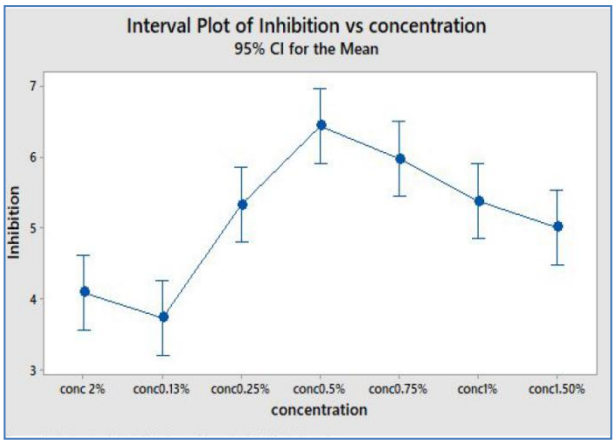


Figure 7:

Table 3:

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
% chitosan present	6	33.43	5.5722	13.75	0.000
Error	35	14.18	0.4052		
Total	41	47.61			

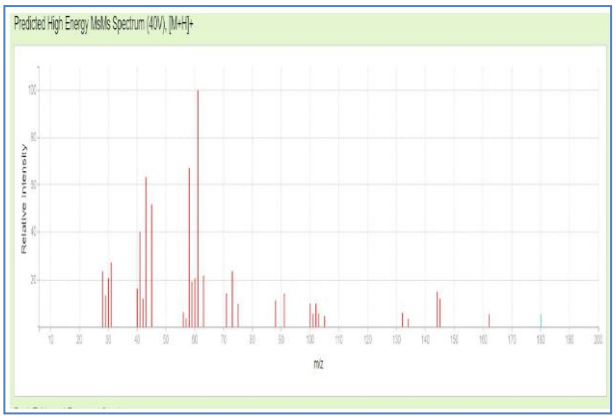
Table 4:

Means

% chitosan present	N	Mean	StDev	95% CI
conc0.13%	6	3.740	0.396	(3.212, 4.268)
conc0.25%	6	5.332	0.374	(4.804, 5.859)
conc0.50%	6	6.445	0.938	(5.917, 6.973)
conc0.75%	6	5.980	0.930	(5.452, 6.508)
conc1%	6	5.383	0.640	(4.856, 5.911)
conc1.50%	6	5.015	0.490	(4.487, 5.543)
conc2%	6	4.095	0.383	(3.567, 4.623)

Pooled StDev = 0.636528

Once the best concentration of chitosan and acetic acid was identified, the reactions involved were studied. Crab shells were dissolved in water in 0.50% concentration which was studied using mass spectroscopy at high energy and the predicted result is given in Figure 8. Results yielded by similar analysis in the presence of 85% acetic acid at medium energy is given in Figure 9. Considering the results in both the figures, a clear shift in the peaks is observed which clearly indicates that a reaction took place. Further, attention weight analysis was performed using spectrophotometer(Figure 10). The figure shows a scattered result at low confidence interval thereby indicating an absence of reaction which is shown by Figure 11. This result contradicts the previous observation. To understand the previous result the solution was kept for an hour.



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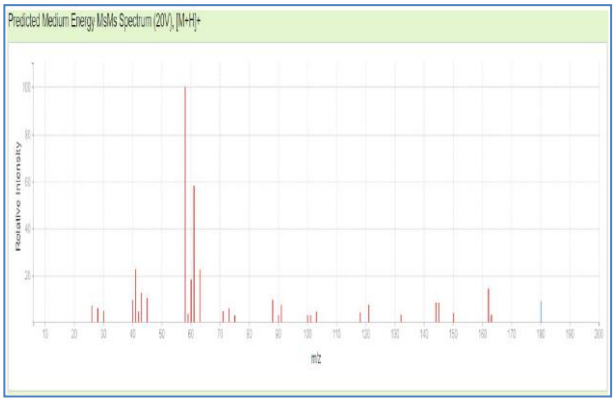

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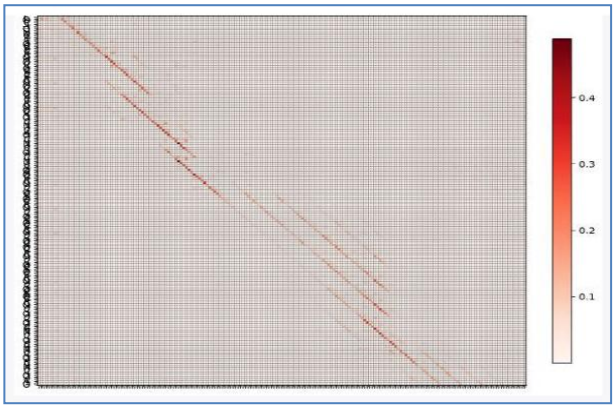

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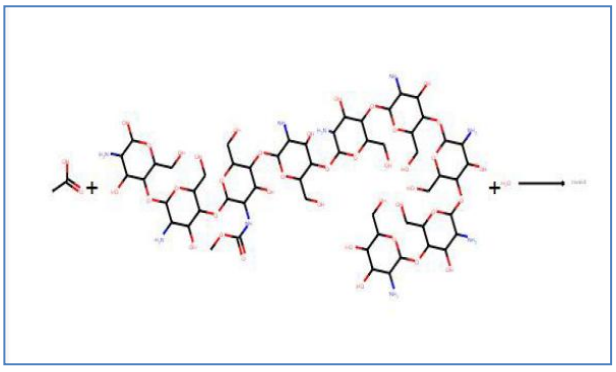
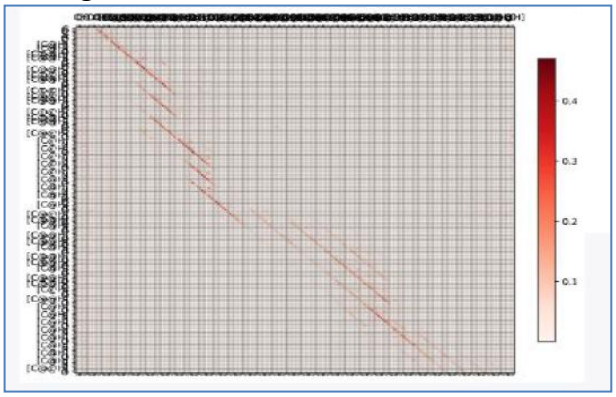


Figure 11:

Attention weight analysis was done once again (Figure 12). The analysis showed less scattered observation indicating that the reaction took place slowly and the isomer of the molecule was involved in the reaction. The reaction for this is given in Figure13. The identified isomer was studied using mass spectroscopy at low energy, again followed by attention weight analysis given in Figure 14 and Figure 15 respectively. The result obtained by attention weight analysis now clearly revealed that the reaction with high confidence interval was took place.

To further verify the reaction a heat map was generated (Figure 16) using 3084 peaks and a total intensity of 150894.5 and maximum intensity 934.5. The curve plotted for observations clearly indicated the excitation of electron with rise of energy and finally the loss of electrons at higher energy followed by no further excitation of electrons. This nature of the curve clearly verifies that protonation reaction took place. The final reaction is given in Figure 17. The molecule in the reaction therefore resulted in the fungal inhibition observed.



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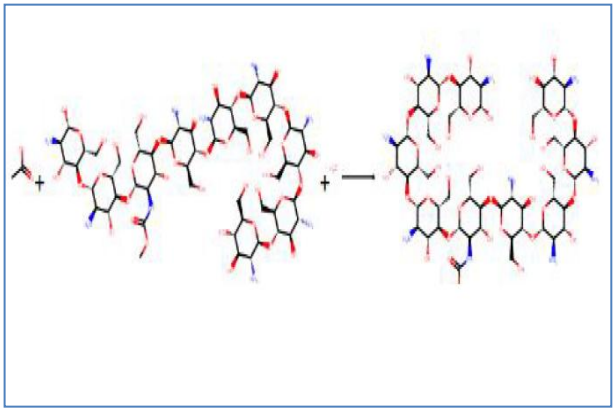

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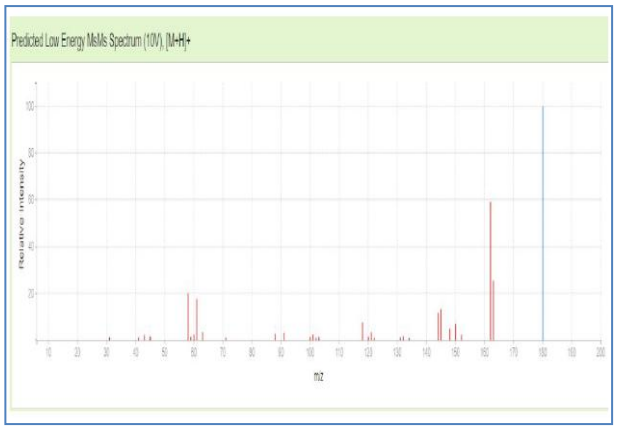

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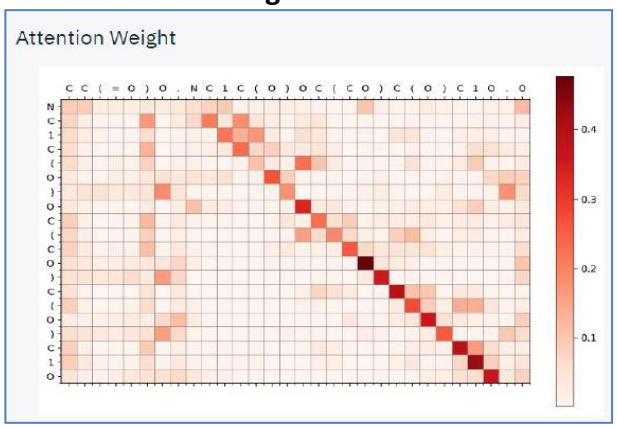

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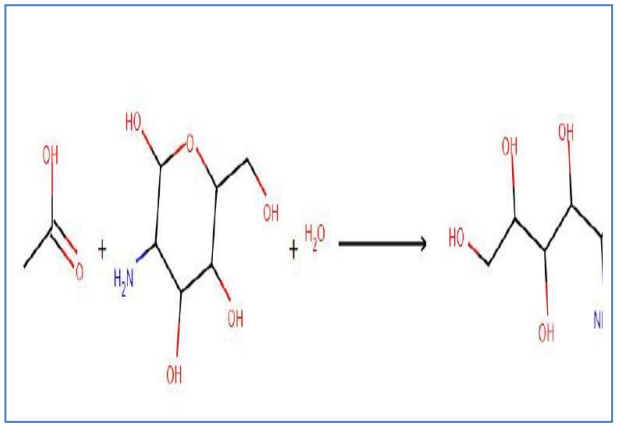

Figure 16:

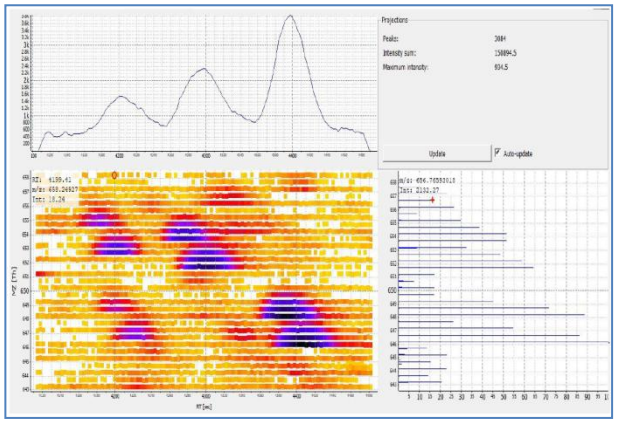

Figure 17:

Result

The first analysis involved finding out the optimum concentration of acetic acid required and the optimum concentration of glucose in the culture medium to maximise inhibition of fungal growth. Comparing the average zone of inhibition, it was evident that larger inhibition zones were observed in samples containing 1 g of chitosan and further analysis showed that maximum inhibition was observed at 85% concentration of acetic acid in 0.25% glucose concentration in culture media. Further, comparing the ideal concentration of chitosan required for maximum inhibition it was observed that largest inhibition zone of diameter 6.455 cm was observed at 0.50% contribution of chitosan. Both individual and day wise analysis yielded the same result thereby identifying 0.50% concentration of chitosan as the ideal concentration.The results were verified statistically using ANOVA which had a p-value 0.00 (<0.05) thereby proving the results to be statistically significant. Mass spectroscopy of 0.50% crab shell in water at high energy and then with 85% acetic acid at medium energy revealed that a reaction took place. Attention weight analysis using spectrophotometer for this molecule showed no reaction taking place. After keeping the solution for an hour the analysis was performed again which now showed that the reaction took place slowly indicating isomerism. The isomer was now studied using mass spectroscopy at low energy followed by attention weight analysis using spectrophotometer again which now clearly indicated that the reaction took place. The reaction was protonation was verified using heat maps and plotting the result to obtain a curve. The obtained curve indicated the loss of electron thereby verifying protonation that was hypothesised.

Conclusion

From the experiment, it can be concluded that 0.50% chitosan in 85% acetic acid was the ideal concentration in which these components acted as efficient fungal inhibitor. The fungal inhibition can be called efficient as this composition gives largest zone of inhibition in 0.25% glucose in culture media, which is a nutrient deficient medium and therefore already has adapted fungi. Mass spectroscopy followed by attention weight analysis using spectrophotometer explained protonation reaction between chitosan and acetic acid and finally explained the structure which was responsible for inhibition of fungal growth in the above mentioned concentration of components.

Reference

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