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Comparison of production rate of alginate by U.V. improved strains of *Azotobacter vinelandii* along with wild type isolates and its FTIR analysis.

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ABSTRACT:*Azotobacter vinelandii* is one of the preferred microorganism for the production of alginate owing to its non-pathogenicity. Alginates are versatile having applications. Bacterial alginates would be the choicest product as compared to seaweed alginate because of its ease in production and maintenance of quality. Seaweed alginate quality cannot be maintained because of seasonal variations and other geographical factors. Studies have been carried out to improve the yield and quality of alginate by using U.V. mutagenised cultures. Improvement in yield has been observed as compared to the non-mutagenised cultures. Maximum production of alginate (4.17±0.08 g/l) was shown by the culture exposed to U.V. radiations at 254 nm for 10 min as compared to unexposed culture (3.50±0.08 g/l) which was determined by its extraction and dry weight measurements. Presence of its components (mannuronic and guluronic acid) were confirmed by FTIR analysis , and its ratio was determined by carbazole reagent (Knutson and Jeans method) which was found to be ranging in between 0.85 to 1.1.

Key words:alginate, *Azotobacter vinelandii*, carbazole, FTIR, U.V. mutagenesis.

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I. INTRODUCTION :

Alginates are linear polysaccharides composed of (1-4)-β-D-mannuronic acid (M) and its C-5-epimer, α-L-guluronic acid (G) , of both technological and scientific interest .[1,2,3] Commercial alginates are extracted from marine brown algae such as *Laminaria digitata, Macrocystis pyrifera* and *L. hyperboreau*. [4,]The monomer composition of these alginates varies andin general one cannot control the qualitative properties of the alginates from sources that involve harvesting in nature. Production of alginate by fermentation using bacteria, like *Azotobacter vinelandii* would be of great advantage for the synthesis of this polymer; however, the maximum alginate concentrations in fed- batch cultures reported so far in the literature is 18.6 g/L and 0.66 g/g respectively.[5,6]Mostly parameters can be carefully controlled in alginates produced by microbes, and such products have a potential for its application in instances where the cost of product is not the most critical parameter. To achieve a good understanding of alginate production in relation to process development, it will be necessary to evaluate large numbers of mutants affected in alginate synthesis, and this requires highly efficient and fast screening procedures for quantification of production levels.[7]The alginate is widely used as thickening, stabilizing, gelling and emulsifying agentin industries like food, textile, paper and pharmaceutical.[8,9] More than half of the total alginate produced world wide is used in food industries suchas ice cream, icy custards, cream and cake mixtures, as well as to keep the contents insuspension in fruit juices.[10] Its immunological use is mainly for the production of monoclonal antibodies from hybridoma cells,[11] stimulating immune cells to secrete cytokines, such as tumor necrosis factor-α, interleukin-1 and interleukin-6.[12] This polymer show interesting feature such as biocompatibility, biodegradability, viscosifying ability and binding with multivalent cations (Jang et al, 1990).[13] Furthur it is also being used as encapsulation agent and therefore have found application in drug delivery and control release system.[14,15] Different bacterial strains such as species of *Azotobacter* and *Pseudomonas* have been reported since long to produce alginate. *A. vinelandii* is more suitable for the biosynthesis of alginate in view of its latent utilization as a food stabilizer.[16] Moreover, the alginate obtained from *Pseudomonas* has poor jellifying ability (Brivonese & Sutherland, 1989).[17] On the other hand considerable pathogenic ability associated with species of *Pseudomonas* has made *Azotobacter* as the preferable genus for the alginate production.[18,19]Enzymes involved in modification of alginate by acetylation, epimerases and lyases are important and contribute to the production of alginates with various desired characteristic properties depending on which it has its applications.[20] Any mutation in genes of these enzymes will either contribute in the quality or the yield of alginate. U.V. radiations cause pyrimidine dimer formation and if this goes unrepaired it may lead to certain unpredictable alterations or changes which may prove to be beneficial in some instances.

II. MATERIALS AND METHODS :

2.1.Cultures used : The reference or the standard culture used is *Azotobacter vinelandii* MTCC 2492. The test cultures were isolated from soil samples of various fields and identified by biochemical tests mentioned in Bergey's Manual of Systematic Bacteriology, vol. 1 & Prokaryotes : A handbook on the biology of bacteria.. (edi.2). The cultures were maintained on Burk's nitrogen free agar slants under refrigeration and stored at -20^{0}C in 20% glycerol stock for further experimental studies.

2.2**.** Physical mutagenesis : For strain improvement, the selected nine cultures alongwith the standard MTCC 2492 strain were cultivated in Burk's medium for 36 hrs. Culture pellet was collected by centrifugation at 8000rpm for 10 min, washed with sterilized distilled water. And was suspended in 10 ml of sterilized distilled water and the dilutions were made upto 10^{-6} for each. The last dilution was selected and 0.1 ml of the aliquot was used to spread on plates and they were exposed to U.V. irradiation at a distance of 15cm in the U.V. cabinet at short wavelength of 254nm for the time intervals of 10s, 20s……100s and further 1min….30 min. The plates were incubated at 30^{0}C along with the control (unexposed). The plates were incubated and checked for the number of colonies and the size of the colonies. Colonies showing maximum polysaccharide production were selected and grown in liquid Burk's medium for production of polysaccharide at 30^{0}C for 72hrs.[21,22] Single mutagenized maximum producing strain from each of the high producing selected wild type were further analysed for checking the difference in M/G ratio.

2.3. Extraction of polysaccharide and separation of biomass : Culture broth samples were centrifuged at 5000 rpm at 10^{0}C for 30 min by using a refrigerated high speed centrifuge to pellet out bacteria with the capsular material. The pellet was suspended in 10mM sodium salt of EDTA for 2 min to solubilise the cell-associated alginate and finally centrifuged at 8,000 rpm for 30 min at 10^{0}C. The pellet was washed with sterile distilled water, centrifuged and dried at 105^{0}C to yield the biomass concentration. The supernatant was collected for extraction of polysaccharide by adding 3 volumes of 95% (v/v) ethanol. Centrifugation was carried out at 7,500 rpm at 4^{0}C for 20 min, the precipitated alginate was washed thrice with 95% ethanol, re-centrifuged and dried in the desiccator and refrigerated. The biomass, alginate pellet were weighed separately.

2.4.Carbazole analysis : The isolated cultures alongwith the standard were preliminary tested for presence of uronic acid content by Carbazole test (Knutson and Jeanes, 1968).[23]The polysaccharide produced from each culture was extracted by using ethanol and the extracted polysaccharide was used for the determination of the ratio of the uronic acids.

2.5.FTIR analysis: The extracted polysaccharide was dried , and by applying KBr technique, FTIR analysis was carried out for the alginate produced by each of the selected five wild type cultures i.e.7,23,25,32,39.

2.6.Statistical analysis: Statistical analysis was done using the version 18 of Minitab and Microsoft Excel 2010.

III. RESULTS AND DISCUSSION :

Table : 1 Production of alginate from the total isolates by shake flask method at 30^{0}C for 72hrs at 150 rpm .

Cultures	Dry cell biomass(g/L)	Alginate(g/L)	Cultures	Dry cell biomass(g/L)	Alginate(g/L)
1.	2.11± 0.01	1.87±0.01	21.	3.09±0.21	2.47±0.32
2.	2.45±0.10	1.86±0.01	22.	2.41±0.48	2.39±0.18
3.	3.15±0.01	2.58±0.13	23.	2.91±0.12	2.74±0.18
4.	3.22±0.02	2.17±0.23	24.	2.00±0.12	1.32±0.16
5.	3.01±0.12	2.47±0.33	25.	3.67±0.23	3.37±0.00
6.	3.35±0.22	3.11±0.11	26.	3.33±0.24	2.34±0.20
7.	3.81±0.22	3.54±0.01	27.	1.99±0..20	1.37±0.10
8.	2.58±0.57	1.99±0.01	28.	2.39±0.31	1.88±0.01
9.	2.52±0.27	1.97±0.02	29.	2.31±0.27	1.67±0.10
10.	2.36±0.26	2.06±0.15	30.	2.70±0.17	2.19±0.30
11.	2.19±0.20	1.76±0.11	31.	2.81±0.16	2.56±0.37
12.	2.26±0.16	1.89±0.01	32.	3.16±0.16	2.77±0.20
13.	3.21±0.13	2.57±0.17	33.	2.63±0.37	2.09±0.14
14.	3.13±0.21	2.36±0.36	34.	2.57±0.37	2.05±0.08
15.	3.42±0.10	2.91±0.15	35.	3.12±0.11	2.47±0.32
16.	2.67±0.54	1.93±0.04	36.	2.35±0.02	1.87±0.07
17.	2.43±0..47	1.31±0.02	37.	3.42±0.09	2.71±0.13
18.	3.23±0..11	2.54±0.34	38.	3.00±0.00	2.44±0.27
19.	3.12±0.11	2.78±0.19	39.	3.29±0.10	2.84±0.14
20.	2.42±0.48	2.38±0.26	40.(std)	2.90±0.02	2.24±0.30

± indicate standard deviation of each value as an average of three replicates.

Table 2 : Production of alginate by different strains of mutagenized cultures exposed for various time intervals.

Cl	Exposed					Unexposed				%inc Y
	T	$B_{(g/L)}$	$A_{(g/L)}$	$A/B_{(g/g)}$	$A/C_{(g/g)}$	$B_{(g/L)}$	$A_{(g/L)}$	$A/B_{(g/g)}$	$A/C_{(g/g)}$	
07	9	3.49±0.01	3.50±0.02	0.845	0.213	4.15±0.53	3.51±0.02	1.002	0.204	42
23	0.5	3.26±0.12	4.15±0.56	1.030	0.239	3.60±0.00	3.71±0.29	1.273	0.218	87.86
25	8	3.52±0.00	3.78±0.12	0.906	0.227	3.96±0.58	3.59±0.02	1.073	0.219	35.24
32	1	3.77±0.12	4.10±0.12	0.987	0.241	4.02±0.15	3.97±0.19	1.087	0.230	45.64
39	4	4.37±0.13	4.17±0.35	1.039	0.243	3.78±0.11	3.93±0.12	0.954	0.234	37.03

Legend : Cl-Cultures used, U-Unexposed culture, E-Exposed culture, T-Exposure time to U.V. in min, B-Dry cell biomass, A-Alginate, %incY-Percent increase in yield, C-Carbon source utilised.

Table 3 : Ratio of mannuronic and guluronic acid.

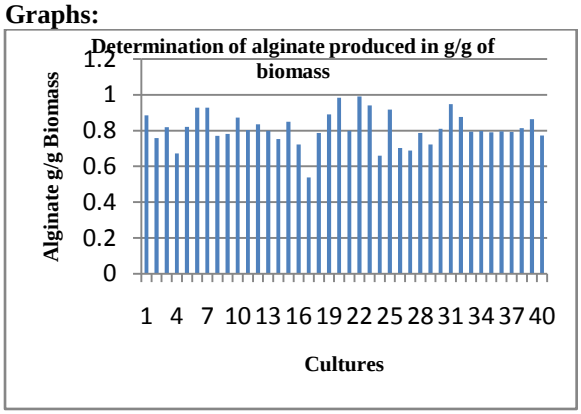
Culture(E)	Ratio	Culture(U)	Ratio
07(Z1)	0.97	07(Z6)	0.96
32(Z2)	1.05	32(Z7)	1.06
23(Z3)	1.1	23(Z8)	1.1
25(Z4)	0.97	25(Z9)	1.02
39(Z5)	0.85	39(Z10)	1.09

Legend : E-Exposed to U.V., U- Unexposed to U.V.

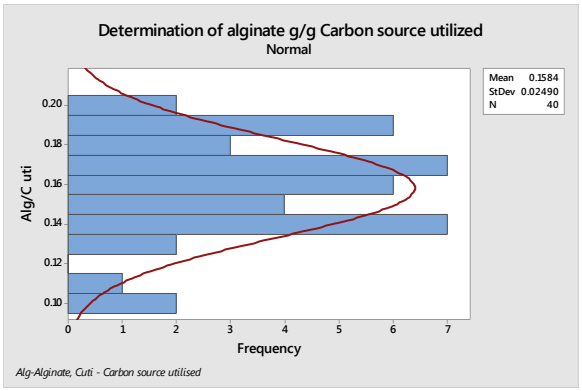
Table 4 : FTIR analysis for determination of functional groups.

Culture	Peaks range	Presence of functional groups
Z1	970-1200, 1600-1730, 3250-3300	C-C, O-H, N-H, C=O, C-H
Z2		
Z3		
Z4		
Z5		

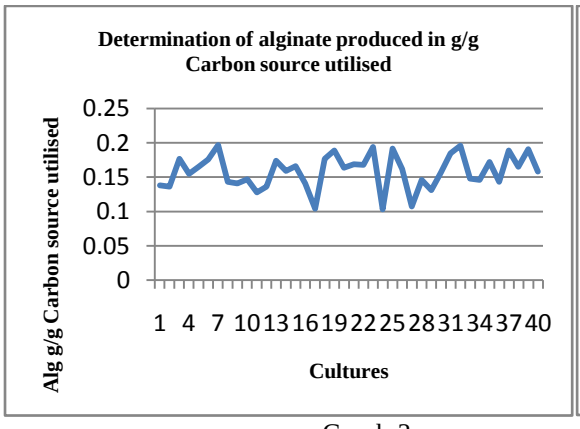
Graphs:



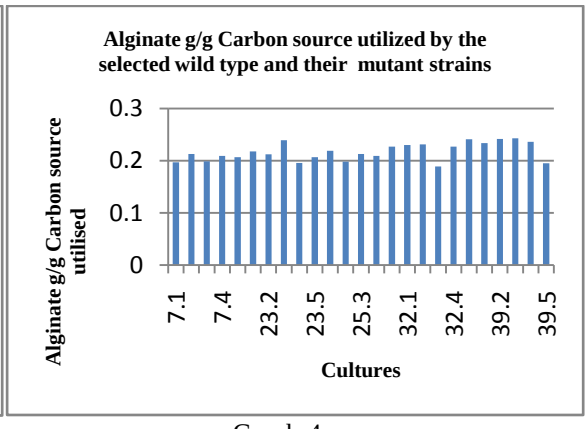
Graph-1



Graph-2



Graph-3



Graph-4

Figures :

Figure 1: Best 05 polysaccharide producers were selected from few U.V. exposed plates.

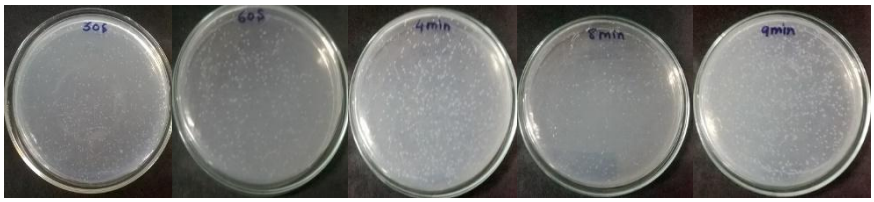


Figure 2 : (2a,2b,2c) Flasks of fermented broth after 72hrs of incubation for samples (Z1-Z10).

Figure : 2a. **Figure : 2b.** **Figure : 2c.**

Figure 3 : Carbazole analysis for the presence of uronic acids.

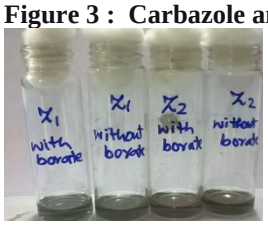


Figure 4 : FTIR analysis of alginate produced by cultures 07, 23, 25, 32, 39 (wild type).

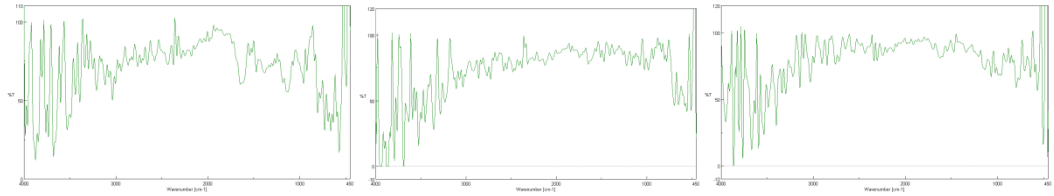


Fig.4a Fig.4b Fig.4c

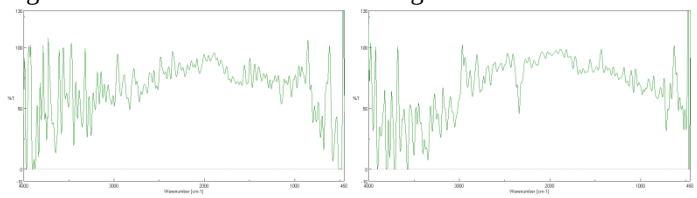


Fig.4d Fig.4e

Soil isolates were compared with the standard reference culture for production of alginate. Table 1 and graph 1 shows the comparative study of biomass and alginate production. Cultures 7,23,25,32,39 were found to be giving higher alginate/Carbon (g/g) utilisation ratio as compared to the other competitive strains on Burk's nitrogen free broth at 30^0C in 72 hrs of incubation time. 40 is the standard reference culture *Azotobacter vinelandii* MTCC 2492. Approximately 50% isolates were found to be giving higher yield than the reference culture. Graph 2 indicates the frequency of alginate producers in various ranges.Graph 3 displays the efficiency of culture in producing alginate in g/g of 'C' source utilized. U.V. mutagenesis was done for the selected cultures by exposing vegetative cells of 24hrs to U.V. radiations at 254nm at a distance of 15 cm for various time intervals ranging from 10…..100sec, 1min….30min (Figure 1). Cultures were found to be surviving and no significant drop in the number of colonies even at exposure to 20 min indicates the effective repair mechanisms. Few colonies showing maximum polysaccharide production from plates exposed at different time periods were selected for production of alginate in liquid Burk's medium (Table 2, Figure 3a,3b,3c). Graph 4 shows the production of alginate by wild type and its mutants,7.1,23.1,25.1,32.1,39.1 are wild type whereas others are mutants.. Maximum producers are 7.2,23.3,25.5,32.5,39.3. It was observed that their was increase in the yield of alginate for some mutagenized strains i.e. 39 mutant on exposure to U.V for 4 min. gave maximum polymer production (4.17g/L) as compared to unexposed culture (3.93g/L) which correlates with the results obtained by [2] i.e. their designated strain UV-66 gave maximum polymer production (4.40g/L) as compared to parent strain (3.35g/L) whereas others showed decline in production rate, which may be contributed to

instability of the mutagenized strains. The results of only the selected high producing strains are displayed in Table 3. Percent increase in yield was calculated and it was observed in the following order 23>32>07>39>25. The chemical test using carbazole reagent for determination of guluronic acid and mannuronic acid content in the extracted alginate indicated that the polymer is having both the residues in various amounts (Table 3,Fig.3). And none of the mutated strain is producing any of the homopolymer as indicated by the M/G ratio. M/G ratio along with molecular weight plays a significant role in its application. Which has been reported earlier by the researchers for example a combination of low molecular weight and high G content produces strongest and biggest alginate beads which has an application in encapsulation of probiotics which remain protected in digestive system. Beads with such characteristic property have small surface area, volume and pore diameter.[25,26,27]Table 4 and fig.4a,4b,4c,4d,4e shows the FTIR analysis of the alginate extracted from selected wild type and mutagenized cultures. FTIR bands were observed in the range of 4000- 400 cm^{-1}. Various peaks were observed as shown in Table 4 which were found to indicate the presence of various functional groups like C-C, O-H, N-H, C=O, C-H. FTIR peaks in the region of 3250-3300 cm^{-1} are due to OH stretching vibrations. And bands at 1200-970 cm^{-1} were due to C-C and C-O stretching vibrations in the pyranose ring and to C-O-C stretching of glycosidic bonds. The relative bands linked to the carbonyl group in alginates can be present as free carboxylic acid (C=O,1726 cm^{-1}) or carboxylate anion (COO^{-},1600-1610 cm^{-1}) form. [17,28,29]

IV. CONCLUSIONS :

U.V. mutagenesis can lead to certain random mutations which may contribute in the increase in yield as compared to the unexposed culture. This may lead to some variation in the guluronic and mannuronic acid ratio which is indicated by carbazole analysis method. FTIR analysis is helpful in rapid identification of the alginate by showing the presence of functional groups. Alginates with various proportion of mannuronic acid and guluronic acid content, molecular weight and viscosity have different biomedical, pharmaceutical and other applications. Alginate production is co-related with the production of dry cell biomass. Percent increase in the yield after mutation is co-related with the utilisation of sugar. Furthur improvement in yield and M/G ratio can be altered by going for chemical mutagenesis i.e carrying out mutation in two stages or employing other strategies of strain improvement. Ratio of M/G was determined by carbazole test. Furthur sophisticated techniques need to be employed for determining the exact content of each.

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