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"Random Mutagenesis of Azotobacter Vinelandii for Enhanced Alginate Production"

Snehalata J.[1]; Dr Jaya K.[2]; Prof. Dr. J. V. Kurhekar [3]
K N Bhise Arts commerce and Vinayakrao Patil Science College, Vidyanagar, Bhosare Kurduwadi[1]
Department of Microbiology, Bharati Vidyapeeth's, Dr Patangrao Kadam Mahavidyalaya, Sangli[2]
ORCID 0009-0004-7109-4005[1]; [3] ORCID 0000-0001-7705-9041

Corresponding Author: **Snehalata Jadhav**

Abstract:
Problem: Alginate, a naturally occurring biopolymer with applications in food, pharmaceuticals, agriculture, and biotechnology, is traditionally produced by Azotobacter vinelandii. However, the natural production capacity of this bacterium remains low, posing a challenge for large-scale industrial extraction. **Approach**: To enhance alginate productivity, random mutagenesis was employed to induce genetic heterogeneity in A. vinelandii. Chemical mutagens were applied, generating diverse mutant strains. These mutants were evaluated through colony morphology, viscosity studies, and fermentation experiments to identify improved alginate producers. **Findings:** Several mutants demonstrated significantly higher alginate yields compared to the wild-type strain. Genetic and biochemical analyses revealed alterations in metabolic pathways, particularly those regulating precursor biosynthesis, polymerisation, and alginate secretion. Overexpression of key biosynthetic genes and redirection of carbon flux toward polysaccharide synthesis contributed to improved productivity. **Conclusion**: Random mutagenesis is shown to be an effective strategy for strain improvement in biopolymer production. The enhanced A. vinelandii mutants provide a promising platform for sustainable, large-scale microbial alginate production, offering economically viable solutions to meet the growing industrial demand for biopolymers.

Keywords: Alginate, Azotobacter vinelandii, Random Mutagenesis, Metabolic engineering, Biopolymer

Introduction

Alginate is widely utilized in the food industry owing to its unique gelling, thickening, and stabilizing properties. Derived mainly from brown seaweeds, sodium alginate is incorporated into a variety of food products to improve texture, consistency, and shelf life. For instance, it is used in ice creams and dairy desserts to prevent ice crystal formation and provide a smooth mouth feel, while in bakery fillings and sauces it functions as a thickener and stabilizer. Alginate-

based gels are also popular in modern gastronomy, especially in "spherification" techniques that create delicate, capsule-like structures for flavor encapsulation. Additionally, alginate films and coatings are being explored as edible packaging materials due to their biodegradability and ability to preserve freshness. Overall, the versatility and safety of alginate make it a valuable ingredient in both traditional food processing and innovative culinary applications.

Alginates are linear polysaccharides comprised of α-L-guluronic acid, its C-5-epimer, and (1-4)-β-D-mannuronic acid, found in marine brown algae and capsular polysaccharides in bacteria (1), (2)(3). Nowadays, brown seaweeds, including Laminaria digitata, Mycrocystis pyrifera, and Laminaria hyperborea, are used to extract commercial alginates (4). Alginate can be produced by a variety of nitrogen-fixing bacteria, including opportunistic diseases such as Azotobacter vinelandii and Pseudomonas aeruginosa. Even though seaweeds are a major alginate source, bacterial alginate is thought to be of higher quality than that of algal alginate. Because of its superior quality attributes, the bacterial alginate is sold for a premium price(5). Various bacterial strains like Azotobacter and Pseudomonas are used for alginate production, but Azotobacter vinelandii is the best candidate due to its noticeable use as a food stabilizer. Due to the pathogenicity of Pseudomonas species, Azotobacter is considered to be the first choice for alginate production (6). When the algal alginateis comparedwith alginate from bacteria, while all other criteria, such as thermostability, gel formation capacity, and the impact of temperature, pH, and NaCl on viscosity, were substantially equal, bacterial alginate has greater pseudo plasticity than algal alginate(7). Because alginate has a negative charge, it can produce a greater viscosity solution in the absence of divalent cations, forming a viscous solution. When divalent or trivalent cations are present, a gel-like structure is formed.

In industrial production, alginate's properties are characterised by their monomer composition, molecular weight, and sequence pattern. There is the possibility to manipulate the molecular weight of alginate according to specific biotechnological or biomedical applications. (8). Alginate has a wide range of industrial applications due to its properties, including stabilising, thickening, viscosifying, and emulsifying. Alginates are also utilised in the paper and textile industries, as well as in the production of ceramics, welding rods, and textile printing. Alginates have also found extensive uses in the biomedical field and food industry,and they are a significant source for the detoxification of wastewater and metals(9), (10).

Alginate is extensively used as a microencapsulation agent in probiotics to enhancetheir viability within the process and as a food additive. In addition, alginates are well known for their biodegradable, biocompatible, and safe biopolymers, so widely used in most medical and pharmaceutical applications as anti-inflammatory agents, radioactive suppressive agents, and wound healing.(2), (11).Alginate is used worldwide in the preparation of creams and icy custards,

cake mixtures, ice cream, and fruit juices to retain the contents in suspension form(4). Alginate plays an important role in the ice cream industry by preventing crystallization as well as shrinkage of ice cream, resulting in homogeneous products. Sodium alginate is used as a stabiliser in salad dressing to avoid phase separation. The same principle is applied in the mayonnaise emulsion phase, water/oil(8).(2) study examined Azotobacter vinelandii's alginate production in batch and fed-batch cultures at a semi-industrial scale. Uncontrolled pH culture yielded the highest volumetric alginate production of 0.98 g/L, while controlled pH culture produced the highest specific alginate production of 0.15 g alginate/g cells. Fed-batch cultivation improved volumetric and specific alginate production to 7 g/L and 0.45 g/g, respectively. (12)study improves Azotobacter vinelandii NRRL-14641strain for enhanced alginate production with the help of shake flask fermentation. A mutant A. vinelandii EMS-45 yields 1.55 times better alginate production than the parent strain. Optimised conditions include nitrogen-rich phosphate-limited medium, pH 7.0, agitation intensity, nitrogen and carbon source.(13) worked on Alginate production by Azotobacter vinelandii mutants altered in poly-β-hydroxybutyrate and alginate biosynthesis. MutantAT268, CNT26, and DM of Azotobacter vinelandii showed reduced production of poly-B-hydroxybutyrate because of phbR and muc26 mutations. All mutants produced 25% less alginate. However, mutant DM had the highest molecular weight for a bacterial alginate, low polydispersity index, and low acetyl content, making it a valuable source for producing improved alginates. (14) worked on bioengineering and molecular strategies to improve alginate and polyhydroxyalkanoate production by Azotobacter vinelandii. Research on Azotobacter vinelandii reveals a complex genetic control of alginate and polyhydroxybutyrate synthesis. Mutants with specific traits, influenced by oxygen tension and growth rate, can produce high molecular mass alginate. A multidisciplinary approach is needed for optimising production. (15) study investigated the optimal conditions for producing bacterial alginate by mutant C-14 of Azotobacter vinelandii NCIB 9068 and also compared its properties with algal alginates. The largest amount of bacterial alginate was obtained in 110 hours, with a viscosity of 18,400 cps. Bacterial alginate was more pseudoplastic than algal alginate, but no notable differences were observed.

In this study, random mutation of Azotobacter vinelandii was used for increased alginate production.

Materials and methods

Soil sample collection

Soil samples were gathered from the botanical garden of K. N. Bhise College, Kurduwadi, District Solapur, India. The sample was kept at 4°C in the refrigerator. The collected samples were thoroughly combined on a piece of clean fabric; lumps were broken up with a wooden pestle and mortar and

allowed to air dry. After being allowed to air dry, the samples were labelled for analysis, placed in glass bottles, and sieved through a 10-mesh sieve.

Isolation of Azotobacter vinelandii

1 gram of soil samples mixed with sterilized saline solution (10 ml) and kept on a rotary shaker incubator at 30°C for 24 hours, the supernatant was filtered and transferred on Ashby's N2 free Mannitol agar containing 20 grams of sucrose, 5 grams of yeast extract, 2 grams of SO_4, 0.2 grams of $MgSO_4$, 1 gram of $CaSO_4$, 0.002 grams of Na_2MoO_4, 0.05 grams of tri ammonium citrate, pH was maintained at 7, incubated for 24 hrs (16), (17).

Screening for alginate producers

Alginate production is carried out by inoculating each isolate in alginate production media having 20.0 g sucrose; 0.6 g (NH4)2SO_4, 2 g Na_2HPO_4; 0.3 g $MgSO_4.7H_2O$ and 6 g yeast extract. All Erlenmeyer flasks of alginate production media were inoculated with each isolate separately. Then incubated at 28°C and 120 rpm for 96 hours(18).

Methods Used for Mutagenesis

UV treatment:

The parent culture of Azotobacter vinelandii S1J was exposed to UV irradiation for different time intervals (0-300 seconds) under a UV lamp (Mineral Light UVS.12, California, USA, λ =253 nm at cycles/S, 220 V). After each exposure mutation cycle culture, the UV lamp screen was kept about 5cm away from the bacterial culture(12).

Ethyl Methyl Sulphonate (EMS) treatment

The random mutation was carried out using the chemical mutagen EMS (sigmaM-880), purchased in liquid form. Mutagenesis of Azotobacter vinelandii was carried out using EMS concentrations of 4-400 mg/ml, causing a 99% lethality rate. Nutrient broth media was used to grow the parental strain overnight until it reached a 108 CFU/ml density. Various concentrations of EMS were added in 5ml of grown cultures aseptically and incubated at 30°C at 150 rpm for 5min. Following incubation, culture broths were centrifuged at 6000 rpm for 15 min. The pellets were washed with sterilised saline twice to remove traces of EMS from bacterial cells. The pellet was resuspended in 10 ml of sterilised phosphate buffer (pH 7.0). This suspension was serially diluted and transferred to skim milk agar plates, and it was incubated at 30°C for 24 h. The number of colonies grown on each plate was counted(19).

Combination of UV and Ethyl Methyl Sulphonate (EMS) treatment

Wild-type culture S1J(CFU~ 10^6) was exposed to UV, the mutated cell culture was centrifuged, and the cell pellet was suspended in PBS (pH 7.4) 1 L of PBS was

made by adding 100 mL of 10X PBS to 00 mL of water. This PBS contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.) These UV-mutated cells were further treated with Ethyl Methyl Sulphonate (EMS) and followed the same procedure mentioned above.

Extraction of Alginate and Partial Purification

The method byClementi (1997) was used for biomass separation and alginate extraction. After incubation 5ml of culture broth was taken in a centrifuge tube, which was pre-weighed. To remove cell-bound alginate, 1 ml of 0.5 M EDTA sodium salt solution and 0.1 ml of 5 M NaCl were added(21). The culture was then centrifuged for 30 minutesat 6000 rpm and at 4°C for biomass separation. The supernatant was kept in an ice bath for cooling, and ice-cold 3 volumes of isopropanol were added to the supernatant. This mixture was kept overnight at 4°C. Alginate precipitation was recovered by centrifugation at 18000 rpm for 30 mins at 4 °C. Precipitates were again dissolved in water, centrifuged, and dried at 80°C for 24 hrs.Alginate estimation was done by dried precipitates(1).

Results and Discussion

Isolation of Azotobacter vinelandii

After 24 hrs incubation period, the colonies were chosen based on their ability to grow quickly. Gummy mucoid, circular, transparent, even margin, convex colonies were observed on Jensen's media with 11 cultures of Azotobacter vinelandii isolated. The morphological and biochemical features of each isolate were identified by using Bergey's Manual of determinative bacteriology.

Screening for alginate production

Shake flask fermentation was used for alginate production; in all isolates of A.vinelandii, alginate production was observed inthe isolate showing the highest alginate production. A maximum alginate producer, S1J, was selected for further study(22).

Mutation study

A study of 15 mutants, including 5 from UV exposure, 6 from EMS exposure, and 4 from combined UV and EMS treatment, found that the highest alginate production was in the S1J-UV-EMS-2 mutant. This suggests that the combined UV+EMS treatment was most effective. UV mutagenesis alone showed better results than EMS alone.

Random Mutagenesis

Results of random mutagenesis of maximum alginate-producing potential Azotobacter strain S1J, which showed maximum alginate production due to the effect of UV and EMS, are presented graphically as per fig. 1,2 and 3 and in Table

1. As a result, total of 15 survivors were selected. Out of these 15 survivors, five were from the UV effect, six were from the EMS effect, and four were due to the combined effect of UV and EMS.

Alginate production by mutagenesis

The alginate production for 15 mutants reveals significant diversity between groups. The UV mutants' output ranged from 3.56 g/L% (S1J-UV-5) to 11.45 g/L% (S1J-UV4), with the majority of values falling somewhere in the middle. The EMS mutants had decreased production rates, ranging from 2.8 g/L% (S1JEMS2) to 9.06 g/L% (S1JEMS6), indicating a more limited alginate output. Interestingly, the combined UV+EMS mutants showed the greatest range of production, from 2.1 g/L% (S1J-UV-EMS-1) to 15.86 g/L% (S1J-UV-EMS-2) (Table 1), indicating a highly varied response to the combined treatment. These findings emphasise the possible impact of mutation type on alginate synthesis, with some mutants exhibiting promising increases, particularly in the UV and UV+EMS groups, and others underperforming. Increasing the sample size may reduce variability and improve the power to detect actual differences between the groups. Additional factors impacting alginate synthesis, such as ambient conditions or experimental techniques and (stability of mutants, should be investigated. Although no significant changes were found in this analysis, the findings are useful for refining future studies and identifying situations that may produce important variances in alginate production.

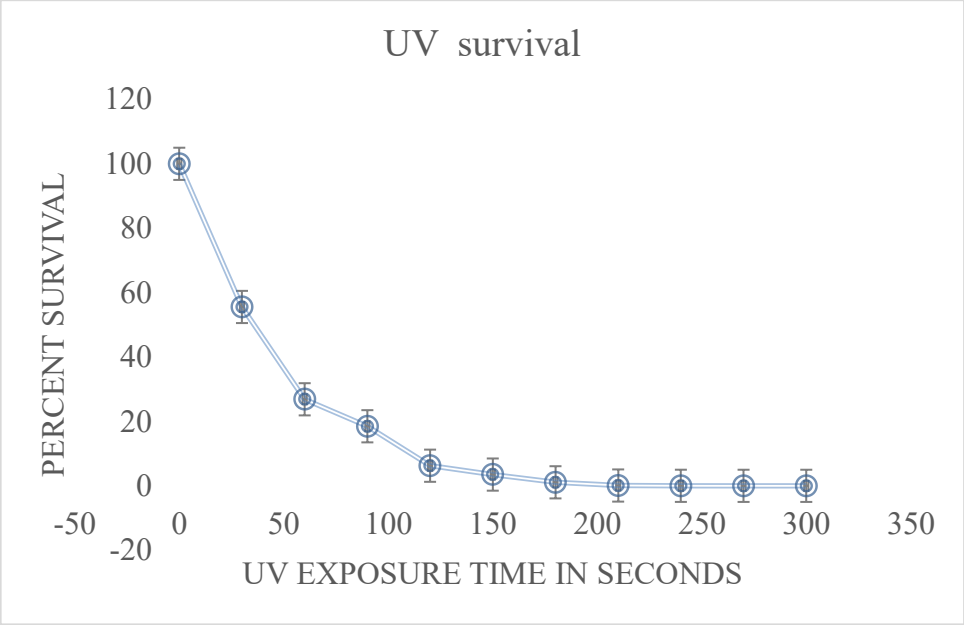

Figure 1. Mutation by UV exposure

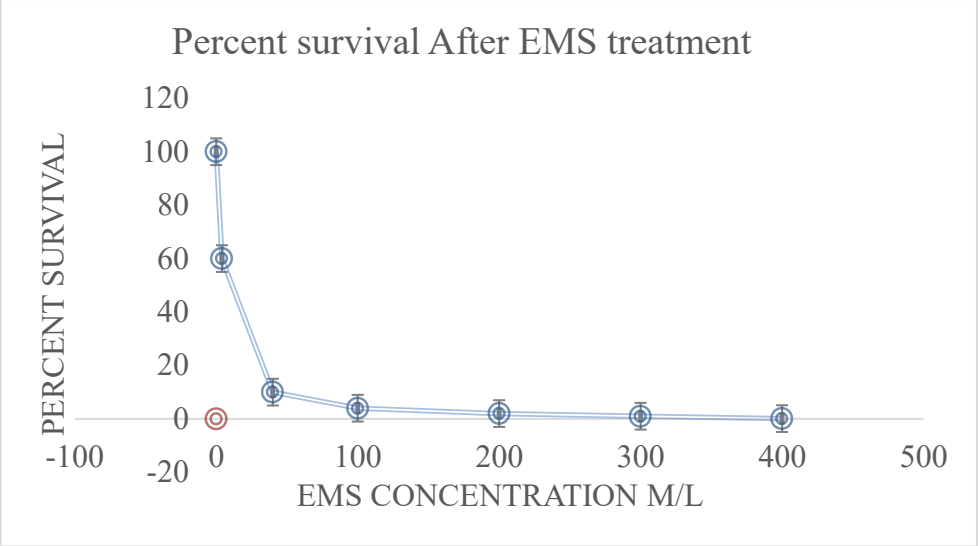

Figure 2. Mutation by EMS (Ethyl Methyl Sulphonate) exposure

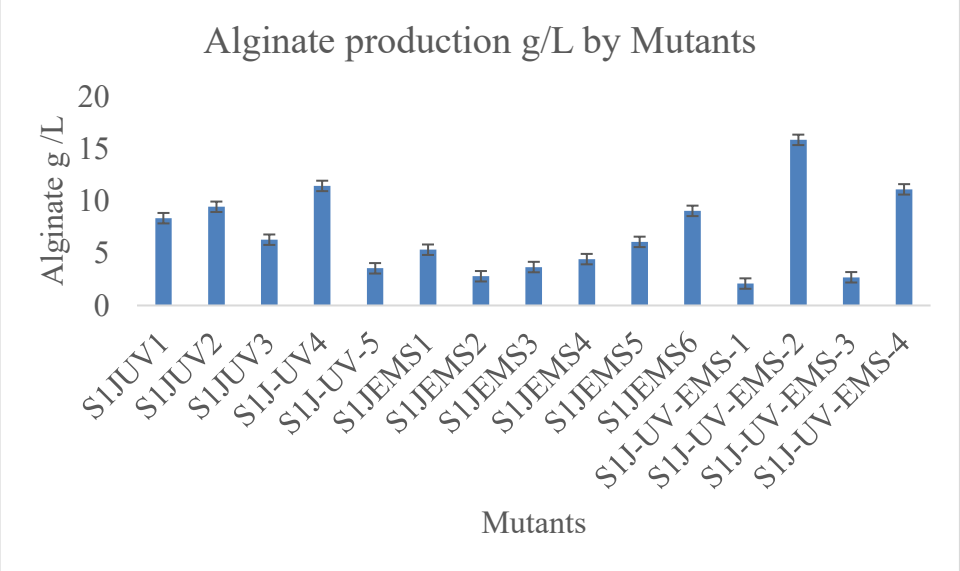

Figure 3. Alginate production by Mutants

Extraction of Alginate and Partial Purification

Pre-weighted tubes were used to collect the supernatant. The pellet obtained from the processes washeddried then weighted for cell biomass. To precipitate alginate, twice the volume of cold isopropyl alcohol (IPA) was added to the supernatant. The supernatant was removed, and the pellet was kept for drying at 105°C for 24 hours. Some of the physical properties and nature of alginate were determined. Alginate showed a creamy to light brown colour with a gummy texture, was soluble in water, and showed insolubility in ethanol and methanol.

Table 1. Alginate production by Mutants

Sr. No.	Mutant	Alginate production g/L
1	S1JUV1	8.36
2	S1JUV2	9.45
3	S1JUV3	6.3
4	S1J-UV4	11.45
5	S1J-UV-5	3.56
6	S1JEMS1	5.34
7	S1JEMS2	2.8
8	S1JEMS3	3.68
9	S1JEMS4	4.44
10	S1JEMS5	6.09
11	S1JEMS6	9.06
12	S1J-UV-EMS-1	2.1
13	S1J-UV-EMS-2	15.86
14	S1J-UV-EMS-3	2.7
15	S1J-UV-EMS-4	11.12

One-way ANOVA resulted in a P value of 0.48. Because the p-value exceeds 0.05, there is no statistically significant difference in alginate production among the UV, EMS, and UV-EMS mutant groups.

Alginate, a vital polysaccharide used in printing, food, pharmaceutical, and textile industries, is produced by bacteria, with their biosynthesis involving molecular mechanisms, and their potential for high-value applications requiring defined material properties(23).

(16) study isolated Azotobacter bacteria from rhizospheric soil, identified them, and screened for alginate production. The potential strain was tested for its potential to produce alginate using an alginate production assay. This study utilised Fourier-Transform Infrared Spectroscopy for characterisation and optimization of parameters. The study found that the Azotobacter (S1J) strain produced the highest alginate biopolymer, supporting commercial synthesis of alginate from bacteria, which is of higher quality than algal alginate. The study examined Azotobacter vinelandii alginate synthesis in batch and fed-batch cultures on a semi-industrial scale. Uncontrolled pH cultures produced the highest volumetric alginate concentration (0.98 g/L), while controlled pH cultures yielded the highest specific alginate (0.15 g alginate per g of cell). Fed-batch cultivation increased both volumetric and specific alginate production by 7 g/L and 0.45 g/g, respectively, similar to results reported by Zalina Othman et al. (2012)(2). Ten Azotobacter chroococcum strains were recovered from soil

samples in Saudi Arabia's El Khurma Governorate, and isolates 1 and 8 produced the most alginate. The maximum yield occurred after 5 days of incubation at 28°C with shaking at 170 rpm. The highest alginate productivity and yield were 102.78 and 37.0%, respectively, at 600 rpm (9). Azotobacter vinelandii A3 was isolated from a cornfield at Abu-Ghraib College of Agriculture. The alginate produced was cream to light brown with a gummy texture, soluble in water but insoluble in ethanol, methanol, and isopropanol. It also binds copper and zinc ions. Similar results were reported by Auhim and Hassam (2013)(1). The study aimed to explore the relationship between alginate production, cell growth, and nitrogenase activity in Azotobacter vinelandii under aerobic conditions. Thirty-five soil samples from Iranian crops were used for microbial isolation and characterisation. The results showed a significant positive Pearson's correlation coefficient of R2 = 0.760 and p ~ 0.02 between nitrogen fixation and alginate production. Bacterial growth, alginate production, and nitrogenase activity were all noticeably correlated. A study on Penicillium janthinellum NCIM 1171 found that mutants with enhanced cellulase production and clear zones on Avicel plates exhibited rapid growth. When the mutants were subjected to larger amounts of 2-deoxy-D-glucose, which was used to hydrolyse Avicel, their FPase and CMCase activity increased by twofold(24). The study concluded that nitrogen fixation and alginate formation are tightly connected under aerobic settings[18]. (18) study discovered that native Azotobacter (S1J) produced 13.05 g/l of alginate in laboratory-scale studies, while mutant S1J-UV EMS 2 produced 15.86 g/l by random mutagenesis, demonstrating effectiveness in alginate synthesis.

Importance of Random Mutagenesis

Because of unexpected genetic changes, limited control over targeted pathways, and a lack of molecular processes generating increased output, random mutagenesis—a technique for strain improvement—has limits in terms of enhancing alginate production in Azotobacter vinelandii. In contrast to contemporary genome editing or systems biology techniques, this renders it labour-intensive, time-consuming, and inefficient.

Proposed Applications of Produced Alginate

Azotobacter vinelandii mutant strains have the potential to boost alginate production by providing high viscosity, gel-forming ability, and biocompatibility. This makes it appropriate for biodegradable films, hydrogels, eco-friendly agricultural products, biomedical applications, food processing, cosmetics, and stabilising agents. Mutagenesis has the potential to reduce production costs while also producing environmentally friendly alginate-based goods.

Conclusion:

Azotobacter vinelandii random mutagenesis boosts alginate synthesis, producing more strains and modifying alginate characteristics. Genetic variation

is essential for improved productivity. Further study could focus on metabolic pathway analysis and genome sequencing, providing a long-term, cost-effective solution for commercial sectors.

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4. Declaration of Generative AI And AI Assisted Technologies in the Writing Process: Chat GPT and other corrective AI has been utilised moderately while preparing this manuscript.
5. Author Contributions: Both the authors contribute to this manuscript equally.
6. Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors. Ethical approval was therefore not required
7. Consent: This article is a review of previously published studies and does not involve any new studies with human participants or animals conducted by the authors. Therefore, ethical approval and informed consent were not required
8. Abbreviations: UV: Ultra violet radiation.
   EMS: Ethyl Methyl Sulphonate
   CFU: Colony forming Units
   IPA: Iso propyl Alcohol

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