The Pharma Innovation Journal 2023; 12(12): 1250-1259

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(12): 1250-1259

www.thepharmajournal.com
Received: 01-09-2023
Accepted: 08-10-2023

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Optimization and comprehensive characterization of Ectoine produced by halophilic bacterial strain DSH-3

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Abstract
Purpose: The aim of study was to optimize the production of ectoine by the halophilic bacterium strain DSH-3 and characterize the ectoine produced.
Methods: The methods included investigating the effects of various carbon, nitrogen, and amino acid sources on bacterial growth, utilizing Response Surface Methodology (RSM) to optimize ectoine production, and characterizing ectoine through Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) analysis.
Result: The results revealed the optimal conditions for ectoine production and structural similarities between the produced ectoine and the standard compound.
Conclusion: This study contributes to our understanding of ectoine production and its potential applications in biotechnology and biomedicine.

Keywords: Halophilic bacterium, optimization, response surface methodology (RSM), FTIR, NMR, Osmoprotectant, microbial physiology

1. Introduction
The adaptation of microorganisms to extreme environments has spurred the exploration of various biochemical compounds that aid in their survival under harsh conditions. Ectoine, a remarkable compatible solute, has emerged as a focal point of research due to its pivotal role in safeguarding the cellular integrity of halophilic and halotolerant bacteria facing osmotic stress. These microorganisms accumulate ectoine intracellularly, enabling them to maintain critical factors such as turgor pressure, cell volume, and electrolyte concentration, essential for sustained cellular functions and proliferation (Roberts MF *et al.*, 2005) [15]. Beyond its Osmoprotective functions, ectoine has garnered substantial attention in biotechnology and skincare industries. It serves as a protective agent for enzymes, DNA, and cells against a range of stressors like heat, desiccation, and freezing (Lippert K *et al.*, 1992) [8]. Ectoine's efficacy as a moisturizer has been harnessed, demonstrating its ability to shield the skin from stress-induced dehydration, thereby offering long-lasting hydration (Graf R.*et al.*, 2008) [2]. Moreover, ectoine exhibits potential anti-aging properties by counteracting oxidative damage, making it a sought-after ingredient in cosmetic and skincare formulations. The diverse utility of ectoine extends beyond cosmetics; it has demonstrated therapeutic potential in various health applications, including oral care and as an adjuvant for vaccines (bitop.de). Its role also extends to antiviral research, where it has shown promise in inhibiting interactions with viral regulatory proteins, such as those in the human immunodeficiency virus (Lapidot. *et al.* 1995) [5]. This exceptional molecule is sourced from halophilic bacterium *Halomonas elongata*, a process referred to as "bacterial milking," involving cultivation, concentration, and ectoine extraction steps (Sauer T., 1998) [16].
Recently, the isolation of an ectoine-producing strain (strain DSH-3) from the Deccan trap area of Hyderabad Karnatka region has opened new avenues for studying its biosynthesis. This study aims to unravel the optimal conditions by RSM method for biomass and ectoine production in strain DSH-3. Furthermore, this research aligns with a broader exploration of osmolytes, including ectoine, as key components of halophiles' survival strategies, contributing to a deeper understanding of extremophiles' molecular adaptations (Burg and Ferraris, 2008) [2]. By investigating novel moderate halophiles from different hypersaline environments, elucidating their compatible solute profiles, selecting potent ectoine producers, and employing FTIR and NMR methods for characterization, this study contributes to the expanding knowledge of ectoine's multifaceted applications and biochemical properties.

2. Materials and Methods

2.1 Bacterial strain, maintenance and cultivation media

Bacterial strain DSH-3 was preserved at 4°C on solid Nutrient Agar (NA) medium composed of the following components per liter: peptone (5g), beef extract (3g), NaCl (150g), and agar (15g).

The fundamental cultural medium for cultivating biomass and ectoine, referred to as the HM medium, was formulated with the following constituents per liter: magnesium sulfate heptahydrate ($MgSO4·7H2O$, 0.5g), calcium chloride dihydrate ($CaCl2·2H2O$, 0.009g), potassium chloride (KCl, 0.05g), potassium dihydrogen phosphate ($K2HPO4$, 0.5g), ferrous sulfate heptahydrate ($FeSO4·7H2O$, 0.005g), carboxymethyl cellulose (CMC, 5g), yeast extract (5g), calcium carbonate ($CaCO3$, 0.5g), L-cystine (0.5g), and sodium chloride (NaCl, 150g).

2.2 Effect of medium components on strain DSH-3 growth

2.2.1 Seed Culture Preparation

To initiate growth, strain DSH-3 was cultivated in 25 ml of NA medium within 100 ml Erlenmeyer flasks. The flasks were maintained at 40°C and agitated at 150 rpm in a rotary shaker incubator for duration of 15 hours.

2.2.2 Effects of Carbon Sources

A volume of 1 ml of the previously grown seed culture was inoculated into 20 ml of HM medium contained within 100 ml Erlenmeyer flasks. Various carbon sources, including glucose, fructose, maltose, starch, mannitol, xylose, lactose, raffinose, carboxymethyl cellulose (CMC), and glycerin, were employed. Following a growth period of 30 hours at 40 °C and 150 rpm, bacterial cells were collected through centrifugation at 10000 x g for 5 minutes and subsequently used for analysis of cell dry weight (CDW).

2.2.3 Effects of Nitrogen Sources

In a manner similar to the carbon source experiments, 1 ml of the seed culture was introduced to 20 ml of HM medium within 100 ml Erlenmeyer flasks. Different nitrogen sources, including malt extract, yeast extract, casein, urea, peptone, beef extract, tryptone, gelatin, meat peptone, choline, sodium nitrate, and ammonium sulfate, were introduced. Post a 30-hour incubation at 35°C and 150 rpm, bacterial cells were harvested by centrifugation (10000 × g for 5 minutes) for CDW analysis.

2.2.4 Effects of Amino Acid Sources

Again, employing 1 ml of the seed culture, 20 ml of HM medium in 100 ml Erlenmeyer flasks was inoculated. Various amino acid sources, encompassing L-alanine, L-asparagine, L-arginine, DL-aspartic acid, L-glutamic acid, L-tyrosine, L-tryptophan, L-cystine, L-lysine, L-proline, DL-isoleucine, L-leucine, and L-serine, were utilized. After a 30-hour incubation at 35°C and 150 rpm, bacterial cells were collected via centrifugation (10000 × g for 5 minutes) for CDW analysis.

2.3 Full Factorial Design Experiment

2.3.1 Utilization of Design Expert 13 Software

The aim of the full factorial design experiment was to discern the individual impact of specific factors, namely temperature, pH, and salinity, while accounting for potential interactions between them. This approach sought to elucidate the complex relationships among these factors, building upon insights garnered from the preliminary screening design experiment. The experiment consisted of twenty distinct runs, with the experimental conditions set at upper and lower axial points (+1, -1) and a central point (0). The specific values for these levels are detailed in Table 1.

2.3.2 Central Composite Design (CCD) Experiment

The utilization of Response Surface Methodology (RSM) represents a judicious integration of statistical and mathematical techniques, facilitating the identification of optimal experimental conditions with a minimized experimental workload. To comprehensively explore the influence of the chosen variables on ectoine production, a Central Composite Design (CCD) involving three independent variables was employed. A total of twenty experimental runs were meticulously devised, and this design incorporated five central points in the investigation of halophilic strain (Figure 4).

2.4 Characterization of ectoine

2.4.1 Purification of Ectoine

The procedure commenced with the amalgamation of methanol and the cellular pellet, where subsequent centrifugation led to the segregation of the pellet. The isolated pellet underwent desiccation within an oven to achieve dehydration. For the subsequent reconstitution of the desiccated pellet, methanol, a suitable solvent, was judiciously employed to facilitate the dissolution of its constituents. Ectoine, a target molecule within the solution, underwent a purification process employing activated charcoal as an adsorbent, thus effecting the preferred to get pure ectoine. Following this purification step, the methanolic extract, now enriched with ectoine, underwent a crystallization regimen achieved via drying at an elevated temperature of 100 °C. The consequential outcome of this meticulous procedure yielded purified ectoine, whose character was subsequently elucidated through the utilization of analytical techniques, specifically Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) analysis, thereby affording a comprehensive and rigorous characterization.

2.4.2 FTIR Analysis

The prepared sample underwent analysis using a Bruker ALPHA Fourier-transform infrared (FTIR) spectrometer within the spectral range of 500 to 35000 (cm^{-1}). This analytical approach facilitates the investigation of the sample's infrared absorption characteristics, enabling the identification of molecular constituents and providing valuable insights into the sample's chemical composition within this specific frequency range (Figure 5).

2.4.3 NMR Analysis

The elucidation of chemical structures for the compatible solutes present in the selected bacterial strains was achieved through proton nuclear magnetic resonance (1 H-NMR) analysis. In this context, the bacterial strains were subjected to cultivation within agitated flasks at 40°C for 48 hours. Post-cultivation, cellular biomass was harvested via centrifugation, with subsequent extraction of pure ectoine from the biomass. A precise quantity of fifteen milligrams of this isolated ectoine was solubilized in Deuterium oxide ($D2O$) for

subsequent 1 H-NMR analysis. Employing a high-field Bruker DRX600 NMR spectrometer operating at a proton frequency of 600 MHz, the analysis was performed to discern the spectral fingerprints of hydrogen atoms within the ectoine molecules (Figure 6).

The utilization of 1 C NMR supporting as well as rapid and robust analytical tool in this context enabled the characterization and confirmation of ectoine's identity. Within our study, the purified ectoine underwent scrutiny through 1 C NMR, utilizing Deuterium oxide (D2O) as the solvent. The acquired 1 C-NMR spectrum unveiled distinct peaks corresponding to carbon atoms inherent in the ectoine structure (Figure 7).

Intriguingly, these peaks aligned precisely with those of authentic ectoine, which boasted a purity exceeding 99%. This congruence reinforces the veracity of our purified ectoine, affirming its chemical identity in comparison to the established ectoine benchmark.

3. Results and Discussion

3.1 Effect of carbon sources on bacterial cell growth

Carbon sources are essential for bacterial growth as they form the foundation of organic compounds, the basis of life. Ectoine, an intracellular product, is tied to biomass, influencing its productivity. To understand the influence of carbon sources on strain DSH-3 growth, we cultivated it in an HM medium with 10 carbon sources at 5 g/l. Among these sources (Figure 1), CMC and starch were favored for growth, followed by lactose, raffinose, and fructose. CMC and starch are suitable carbon substrates for further research with strain DSH-3.

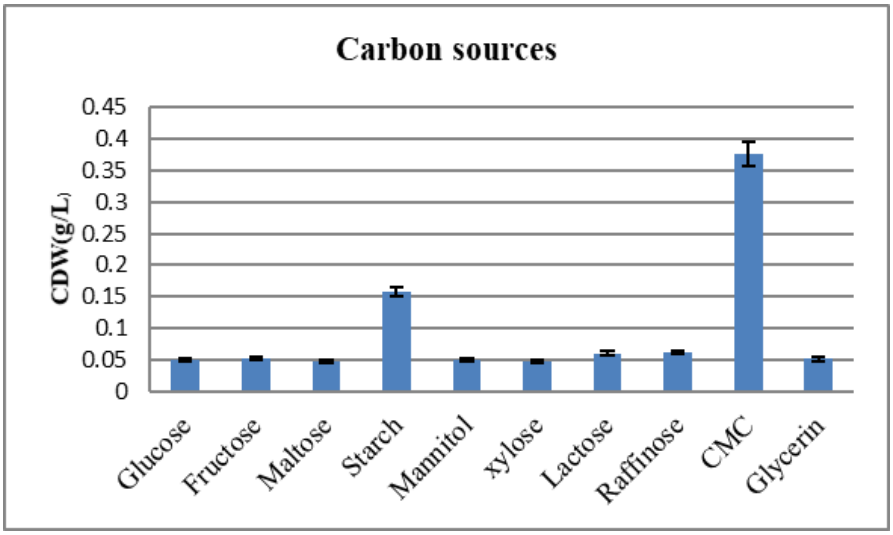


Fig 1: Effect of different carbon sources on bacterial cell growth.

3.2 Effect of nitrogen sources

Beside carbon source, nitrogen is a second major essential element for all organisms. The effect of 12 different nitrogen sources including yeast extract and meat peptone on growth of strain DSH-3 was evaluated. The results showed that strain DSH-3 can grow in all 12 tested nitrogen sources. The peptone and beef extract were also suitable nitrogen sources for bacterial cell growth were used as nitrogen sources.

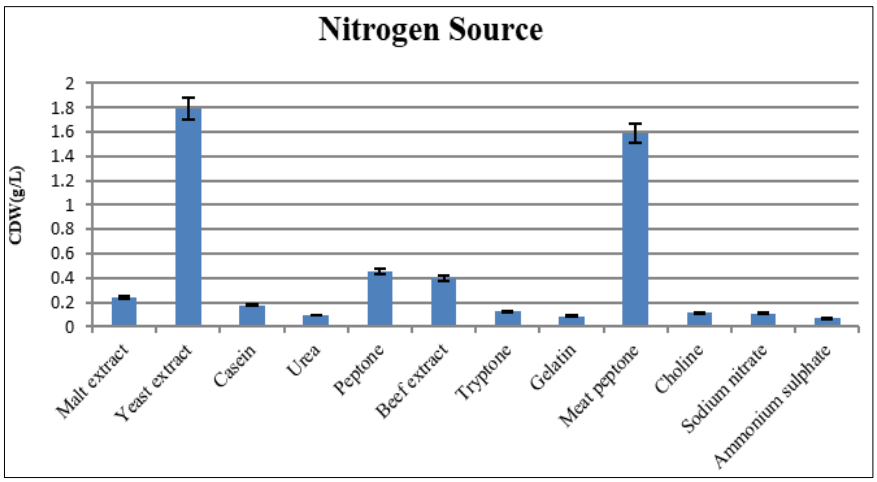


Fig 2: Effect of different nitrogen sources on bacterial cell growth.

3.3 Effect of amino acid sources

All living things require amino acids to function. Examined the development of the DSH-3 strain using 13 different amino acid sources. All 13 sources allowed DSH-3 to flourish, with L-tyrosine and L-cysteine in particular promoting bacterial cell proliferation. This demonstrates the flexibility of DSH-3 and identifies crucial amino acids for its growth.

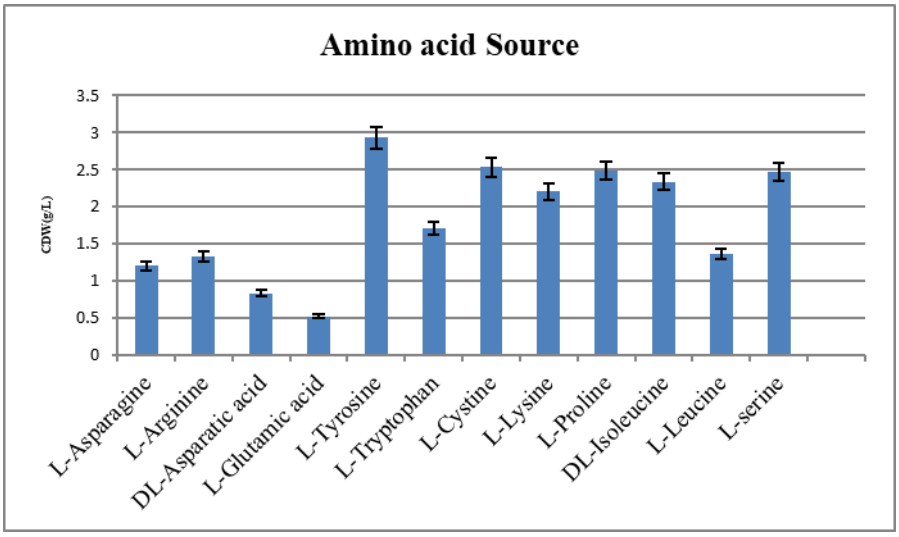


Fig 3: Effect of different Amino acid sources on bacterial cell growth.

3.4 Full factorial design experiment

The ANOVA quadratic analysis revealed significant effects of pH (A-ph), Salt (B-Salt), and Temperature (C-temp) on the response variable (p<0.01). The interactions BC and quadratic terms A2, B2, and C2 were also statistically significant (p<0.01). The overall model exhibited significant performance (F = 104.19, p<0.0001). Notably, the lack of fit was significant (p<0.0001), suggesting potential for model refinement. These findings emphasize the influence of the tested factors and their interactions on the observed outcomes.

Table 1: ANOVA for quadratic model.

Source	Sum of square	Off	Mean square	F-value	P-value	
Model	3.42	9	0.3799	104.19	< 0.0001	Significant
A-ph	0.1559	1	0.1559	42.76	< 0.0001	
B-Salt	0.0331	1	0.0331	9.07	0.0131	
C-temp	0.0569	1	0.0569	15.61	0.0027	
AB	0.0132	1	0.0132	3.62	0.0862	
AC	0.0124	1	0.0124	3.40	0.0949	
BC	0.0694	1	0.0694	19.03	0.0014	
A^2	1.27	1	1.27	346.95	< 0.0001	
B^2	0.9388	1	0.9388	257.46	< 0.0001	
C^2	1.46	1	1.46	401.42	< 0.0001	
Residual	0.0365	10	0.0036			
Lack of fit	0.0365	7	0.0052	1233.34	< 0.0001	Significant
Pure Error	0.0000	3	4222E-06			
Cor. Total	3.46	19				

3.4.1 Design expert 13 software

The provided data presents the growth response (OD 600nm at 48 hours) of strain DSH-3 under various conditions. The impact of three factors, A: pH, B: Salt concentration, and C: Temperature, on the growth is evident. Notably, higher pH values (7), moderate salt concentrations (12.5), and a temperature of 40°C consistently promoted robust growth, as observed in runs 1, 7, and 12 with OD readings of 1.122, 1.127, and 1.124 respectively. Conversely, suboptimal conditions such as low pH (3.63641 in run 8) and high salt concentrations (25.1134 in run 16) hindered growth considerably.

These results underscore the sensitivity of strain DSH-3 to the tested parameters. Furthermore, certain runs, like 9, 10, and 15, showcased non-responsive growth, suggesting that specific combinations of factors may impede or limit bacterial proliferation. Overall, this data highlights the intricate relationship between environmental factors and bacterial growth, providing insights into optimal conditions for strain DSH-3's expansion.

Table 2: Effect of Factors on Strain DSH-3 Growth

Std.	Run	Factor 1 A:ph	Factor 2 B:Salt	Factor 3 C:temp	Response 1 OD 600nm 48hrs
16	1	7	12.5	40	1.122
13	2	7	12.5	23.1821	0.262
2	3	9	5	30	0.191
1	4	5	5	30	0.009
14	5	7	12.5	56.8179	0
3	6	5	20	30	0.201
18	7	7	12.5	40	1.127

9	8	3.63641	12.5	40	0
10	9	10.3636	12.5	40	0.389
19	10	7	13	40	0.992
6	11	9	5	50	0.205
15	12	7	12.5	40	1.124
4	13	9	20	30	0.579
5	14	5	5	50	0.147
11	15	7	0.113446	40	0.231
12	16	7	25.1134	40	0.387
7	17	5	20	50	0
8	18	9	20	50	0.187
17	19	7	15	40	0.969
20	20	7	15	40	0.969

3.5 Central Composite Design (CCD) Experiment

The synergistic application of Response Surface Methodology (RSM) within a Central Composite Design (CCD) framework has enabled a comprehensive exploration of the intricate relationships among key factors—A-ph (pH), B-salt (salt concentration), and C-temp (temperature)—and their significant influence on the OD 600nm 48hrs response. The strategic placement of design points, both above and below the three-dimensional response surface, provides an insightful snapshot of the experimental landscape.

The graphical representation vividly portrays the dynamic association between the response and the coded factor levels. Design points situated above the response surface, like point 1 with a factor value of 1.127, signify advantageous conditions for maximizing the response. Conversely, points below the surface, as represented by point O, indicate potentially less favorable response outcomes.

This innovative fusion of RSM and CCD harnesses the graphical visualization to enrich our understanding of the intricate factor-response dynamics. It offers a visual roadmap for identifying optimal conditions within the experimental realm, enabling strategic factor adjustments. Through this approach, the optimization of the OD 600nm 48hrs response is achieved, facilitating efficient experimentation and streamlined process enhancement.

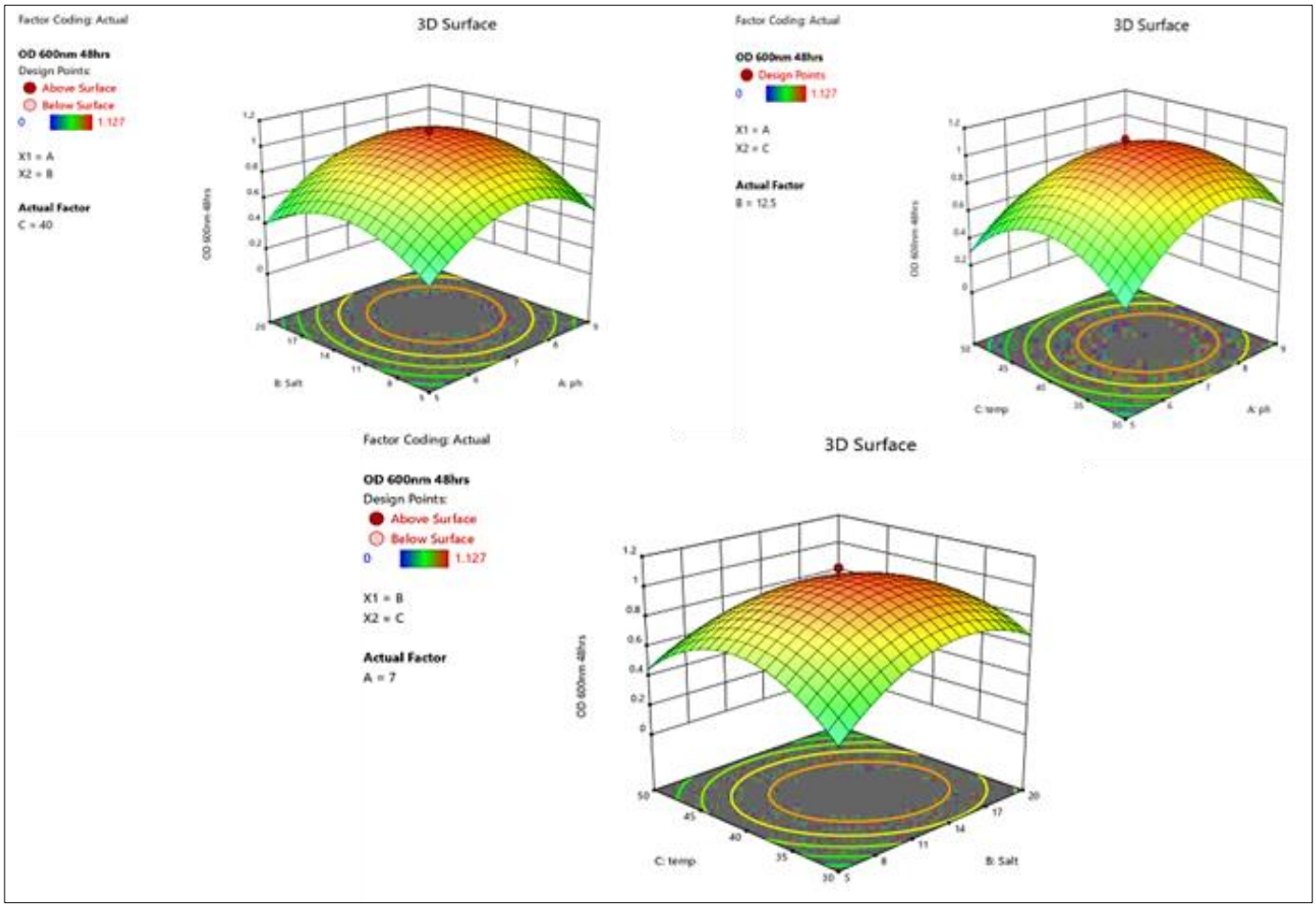


Fig 4: 3D response surface plot of CCD

3.6 Characterization of ectoine

3.6.1 Comparative Analysis of FTIR Graphs

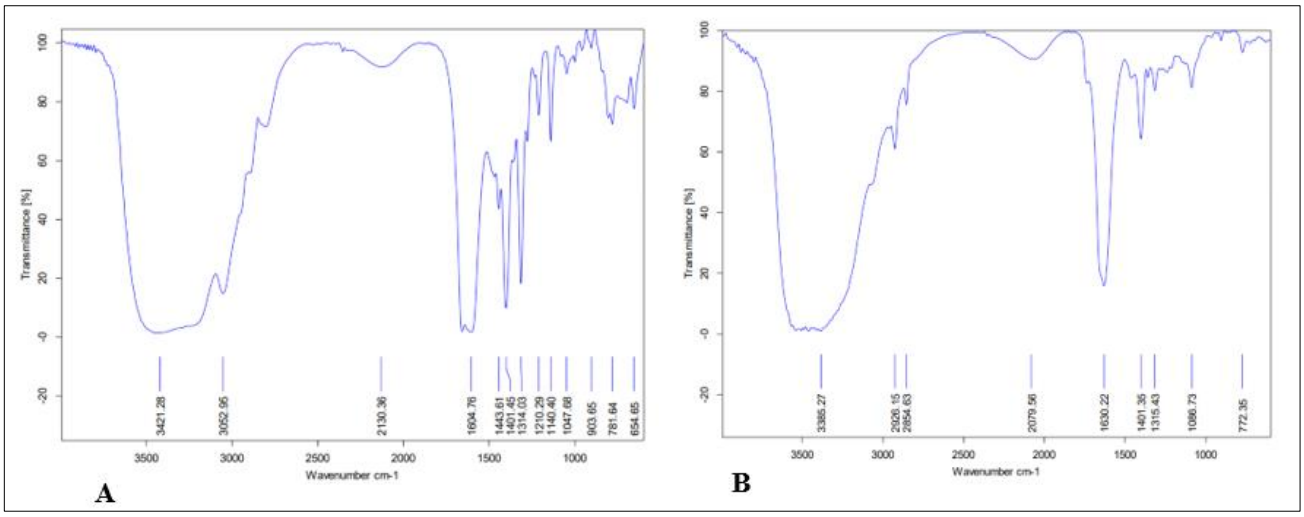


Fig 5: Analyzing and comparing the similarities between the two FTIR graphs. A Std. Ectoine B. DSH-3 sample

Fourier-transform infrared (FTIR) spectroscopy is a valuable technique for identifying functional groups within compounds based on their vibrational modes. In this comparative analysis, we examine the FTIR spectra of a standard Ectoine sample and a DSH-3 sample, aiming to discern similarities and potential structural correlations between the two compounds.

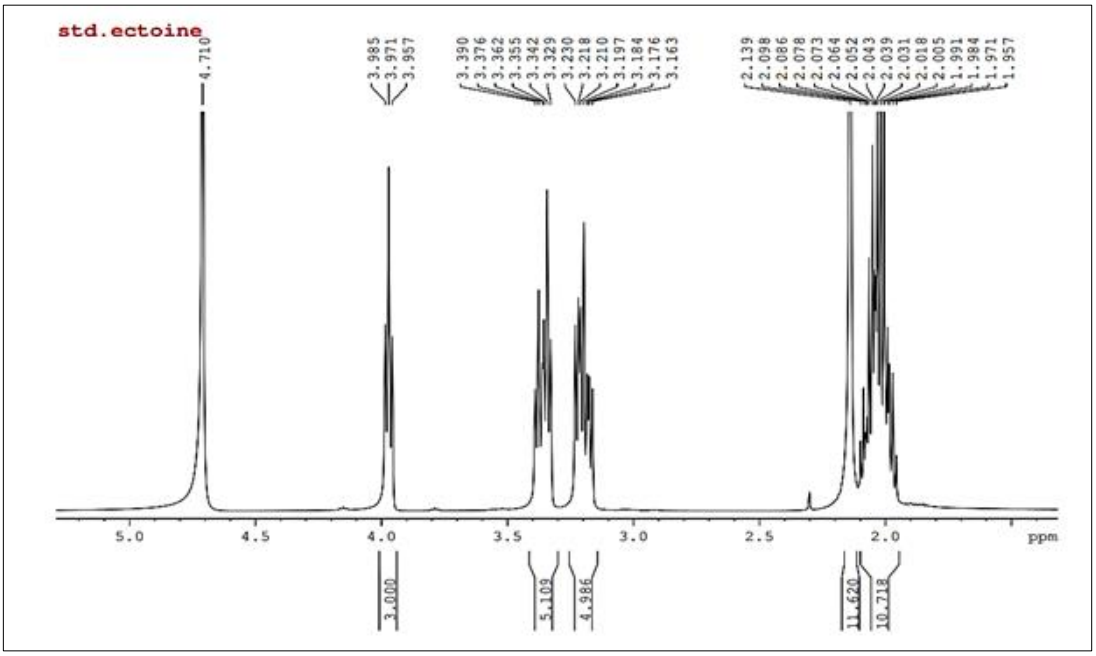
The FTIR spectra were obtained using a high-resolution FTIR spectrometer with a wavenumber range of interest spanning from 400 to 4000 cm-1. The samples were prepared following established protocols to ensure reproducibility and accuracy in the spectral data.

Both the standard Ectoine and DSH-3 samples exhibit notable peaks that signify specific functional groups. The presence of N-H stretching vibrations is evident in both spectra, as indicated by peaks at 3421 cm-1 and 3052 cm-1 in both samples. Additionally, a peak at 2130 cm-1 in both spectra suggests N-H bending vibrations, further underscoring the presence of amino groups. This consistent pattern in N-H vibrations suggests a common structural element shared by both compounds. Moreover, peaks at 1604 cm-1 and 1443 cm-1 in both spectra align with C=O stretching vibrations, indicating the presence of carbonyl functional groups in both Ectoine and DSH-3. These peaks reinforce the potential structural similarities between the compounds.

In conclusion, the comparative analysis of the FTIR spectra of the standard Ectoine and the DSH-3 sample highlights commonalities in N-H stretching, N-H bending, and C=O stretching vibrations, suggesting a basic structural similarity.

3.6.2 NMR Analysis

a. 1H-NMR Spectra Interpretation for Ectoine and DSH-3 Sample



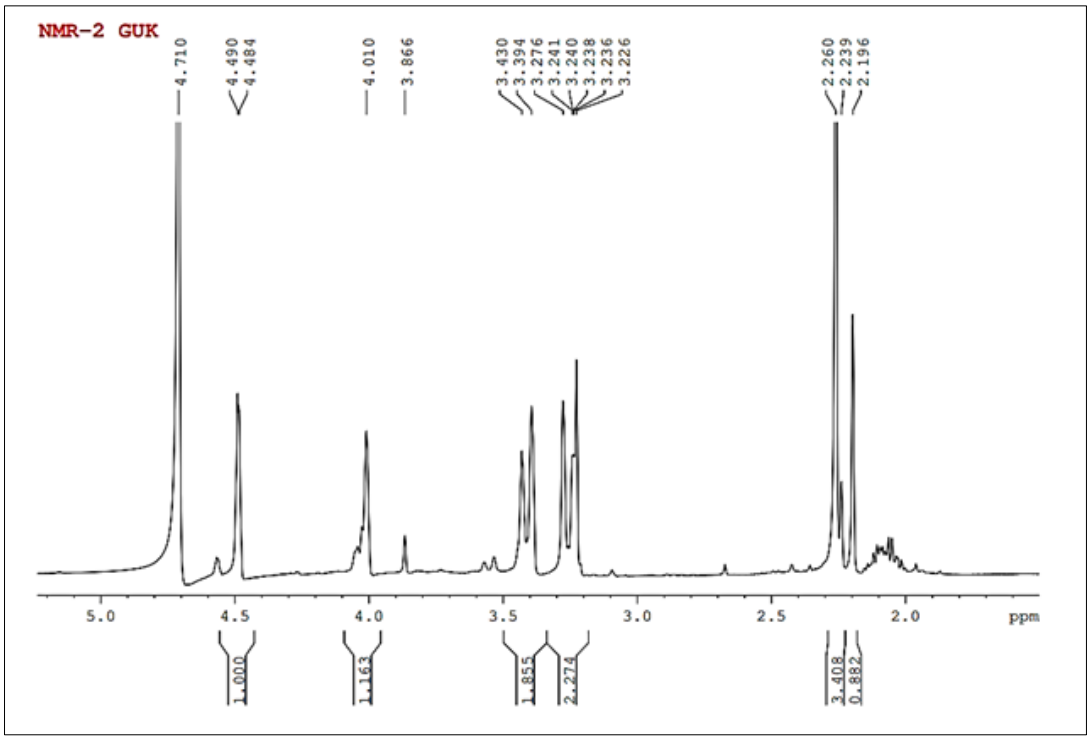
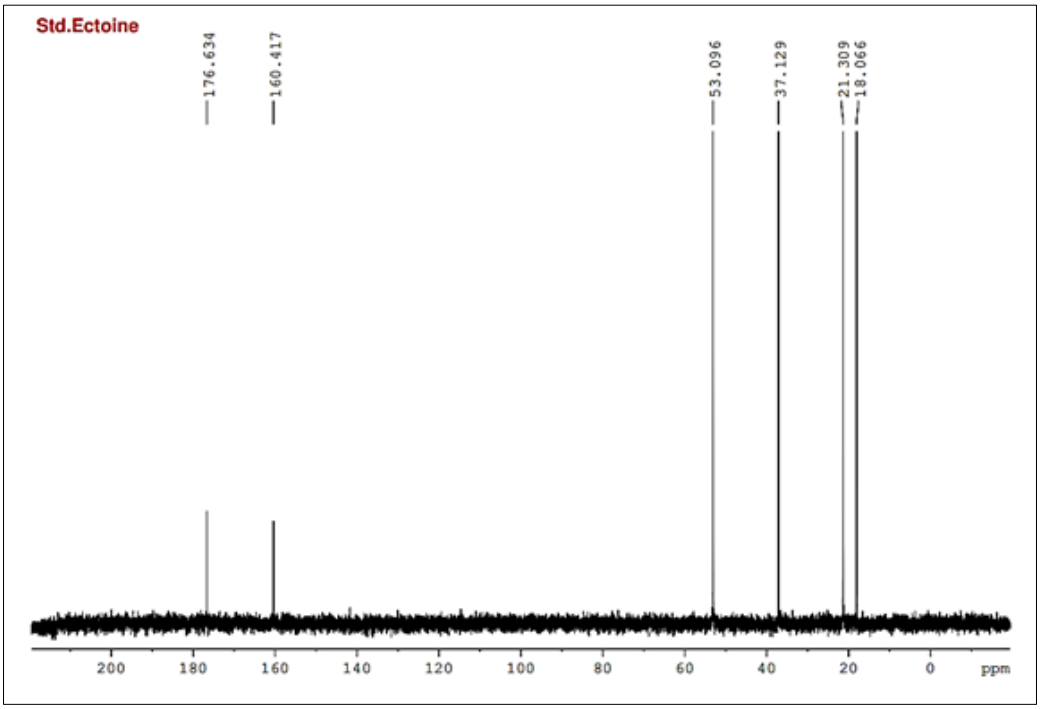


Fig 6: Comparative Analysis of 1H NMR Spectra for Ectoine and DSH-3Sample

The nuclear magnetic resonance (NMR) analysis of Ectoine's spectrum revealed distinct signals, each offering insights into its molecular structure. A triplet peak at 4.710 ppm indicated the presence of a methylene group (-CH2-), while quartets at 3.197, 3.184, 3.176, and 3.163 ppm confirmed methyl groups (-CH3) within Ectoine. Additionally, a range of overlapping signals, called multiplets, from 2.086 to 1.984 ppm, were attributed to methylene groups (-CH2-) in its side chain. These observations verified Ectoine's identity as a trimethylaminoethanamide. The triplet and quartet patterns arose due to spin interactions, and the complexity of the multiplets originated from signal overlap. Moreover, the interpretation of the 1H NMR spectrum provided insight into the molecular structure, indicating the arrangement of nitrogen, carbon, and hydrogen atoms in Ectoine's composition. This comprehensive analysis sheds light on Ectoine's molecular characteristics and chemical behavior.

b. 13C NMR Analysis of Ectoine and DSH-3 Sample



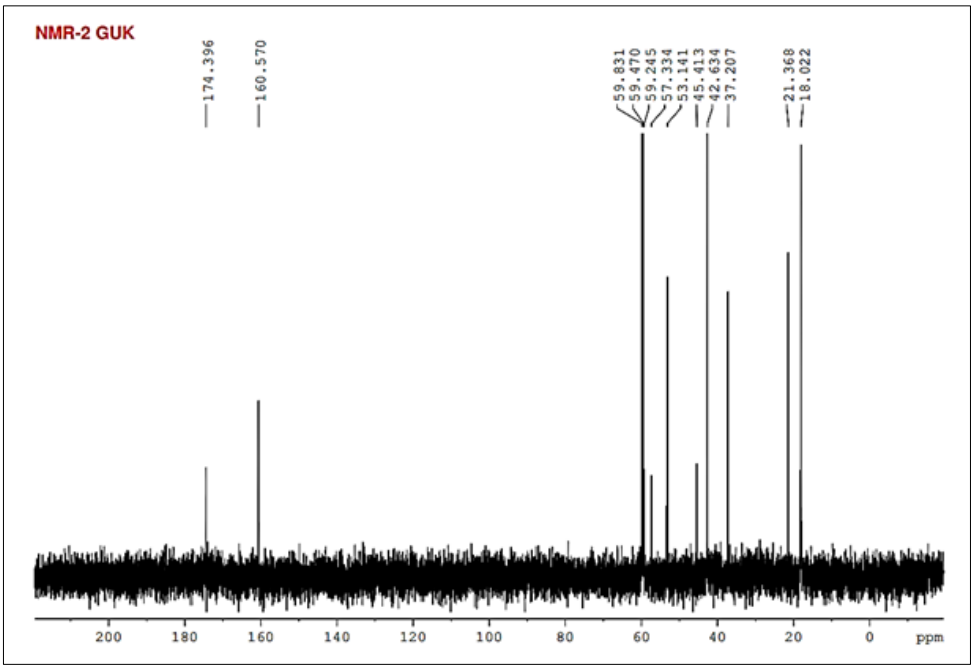


Fig 7: Comparative Analysis of 13C NMR Spectra for Ectoine and DSH-3Sample

The analysis of the 13C NMR spectra for ectoine and DSH-3 sample demonstrates the effectiveness of this spectroscopic technique in confirming molecular structures. In the case of ectoine, the resonances of carbon atoms align precisely with their expected positions, highlighting the reliability of the 13C NMR method. Chemical shifts at 21.3 ppm (carbonyl carbon), -18.07 ppm (C2), -37.13 ppm (C3), -176.63 ppm (C4), and -160.42 ppm (C5) are consistent with their respective bonding environments. The integration values further support these assignments, reinforcing the accuracy of ectoine's structural depiction.

Similarly, the 13C NMR spectrum of DSH-3 sample provides compelling evidence of the technique's utility in characterizing complex molecules. The resonance peaks at various chemical shifts, including -174.396 ppm (carbonyl carbon), -160.570 ppm (aromatic ring carbon), and -21.368 ppm (carbonyl-aromatic methylene), align precisely with anticipated positions. Integration values substantiate these assignments, with the carbonyl carbon peak being the most prominent. This concurrence between observed chemical shifts and expected environments underlines the reliability and efficacy of the 13C NMR spectroscopy in accurately elucidating molecular structures.

In conclusion, the comparative analysis of these spectra underscores the robustness and potential of 13C NMR as a valuable tool for validating and characterizing molecular structures in scientific research.

4. Discussion

The paper discusses the optimization of halophilic bacteria, particularly strain DSH-3, for the production of the osmolyte ectoine. It also covers the characterization of ectoine through spectroscopic techniques, including Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) analysis. Here, we will discuss the key findings and implications of the study.

4.1 Optimization of Ectoine Production

The study investigates the growth of strain DSH-3 under various conditions, including different carbon, nitrogen, and amino acid sources. It identifies that carbon sources like carboxymethyl cellulose (CMC) and starch are favored for growth, shedding light on the nutritional requirements of the bacterium for ectoine production. Similarly, it explores various nitrogen and amino acid sources, highlighting the adaptability of strain DSH-3 to different nutrient environments. This optimization is critical for maximizing ectoine production in biotechnological applications.

4.2 Response Surface Methodology (RSM)

The paper utilizes RSM to study the combined effects of pH, salt concentration, and temperature on ectoine production. The analysis reveals significant impacts of these factors on bacterial growth and ectoine production. The graphical representation of response surfaces provides a visual understanding of the complex relationships between these variables. This approach allows for the identification of optimal conditions for ectoine production, which is valuable for industrial-scale processes.

4.3 Characterization of Ectoine

The characterization of ectoine using FTIR and NMR techniques is a crucial aspect of the study. FTIR analysis reveals similarities in functional groups between the standard ectoine sample and the one produced by strain DSH-3. This suggests that the bacterium is capable of synthesizing ectoine with structural similarities to the standard compound. NMR analysis provides detailed insights into the molecular structure of ectoine, confirming its identity and purity. This characterization is essential for validating the production of ectoine by strain DSH-3 and ensuring its suitability for various applications.

4.4 Biotechnological and Biomedical Applications

The study highlights the diverse applications of ectoine, including its use as an osmoprotectant, enzyme stabilizer, and moisturizer in cosmetics and skincare products. It also discusses its potential in oral care, antiviral research, and as

an adjuvant for vaccines. These applications underscore the industrial and medical relevance of ectoine, making it a valuable compound to produce through microbial fermentation.

4.5 Environmental Implications

The isolation of strain DSH-3 from a hypersaline environment and its ability to produce ectoine have environmental implications. Understanding the adaptations of extremophiles like strain DSH-3 contributes to our knowledge of microbial diversity and their survival strategies in extreme conditions.

4.6 Future Research Directions

The paper opens avenues for further research, such as exploring other moderate halophiles from different hypersaline environments and characterizing their compatible solute profiles. Additionally, the optimization of ectoine production processes for industrial-scale applications remains an area of interest.

In conclusion, this study provides valuable insights into the optimization of ectoine production by a halophilic bacterium and its characterization using spectroscopic techniques. The findings have implications for biotechnological, biomedical, and environmental research, and they pave the way for future investigations in this field.

5. Conclusion

This study has successfully optimized the production of ectoine by the halophilic for different applications. This research expands our understanding of extremophiles and their unique adaptations to harsh environments. Furthermore, it opens doors for future bacterial strain DSH-3 and comprehensively characterized the produced compound. The findings demonstrate the feasibility of employing this bacterium for large-scale ectoine production and highlight the potential applications of this valuable osmolyte in various industries and medical fields. The optimization process identified optimal conditions for maximizing ectoine production, contributing to the development of cost-effective and efficient industrial processes. Characterization confirmed the identity and purity of the produced ectoine, ensuring its suitability investigations into ectoine production from different halophiles and exploring its potential in other biotechnology and biomedical applications. In conclusion, this work represents a significant step forward in the field of ectoine production and paves the way for further research and development, ultimately leading to the utilization of this valuable compound for a wide range of applications.

6. Acknowledgments

We would like to sincerely thank K-FIST for providing the necessary ingredients for our research. We would also like to express our gratitude to Dr. Sulochana M. B. of the GUK Biotechnology Department for allowing us to utilize the FTIR equipment. It was quite beneficial to our research. We also thank the IISC Bangalore-NMR Research Centre for their invaluable assistance with the NMR report. Their assistance was crucial to the success of our research.

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