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BIOMOLECULE PRODUCTION BY MICROORGANISMS ISOLATED FROM SALINE ENVIRONMENTS

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Abstract

Saline environments, characterized by extreme conditions, are unique habitats that harbor diverse microorganism communities capable of synthesizing biomolecules, such as extracellular hydrolytic enzymes and carotenoid pigments with significant industrial potential. These biomolecules are important in microorganisms' survival and adaptation to harsh environments. The present study aimed to isolate several new biomolecule-producing microorganisms from two salt wells (Curmătura and Băicoi, Prahova County, Romania). The strains NC18, NC21, NC28, and SB1 were isolated using a selective agar medium supplemented with 3.4 M sodium chloride (NaCl). Specifically, NC18, NC21, and NC28 were obtained from Curmătura, while SB1 was isolated from Băicoi. As a result of their ability to grow on agar medium with 1–5 M NaCl, all the new isolates were included in the extreme halophilic organisms. Based on their phenotypic and molecular characteristics, all these strains were included in the domain Archaea. The four isolates NC18, NC21, NC28, and SB1 were further assessed for their ability to produce extracellular hydrolytic enzymes, including lipase, protease, amylase, cellulase, xylanase, and pectinase, as well as carotenoid pigments. Due to their ability to produce a range of bioactive compounds, the halophilic isolates present promising opportunities for diverse biotechnological applications, such as industrial enzyme production, and the development of bio-based products.

Key words: *carotenoids, exoenzymes, halophiles, saline environment.*

INTRODUCTION

Halophilic Archaea, primarily belonging to the family *Halobacteriaceae*, are extremophiles that thrive in hypersaline environments, where high salt concentrations inhibit the growth of most other organisms. Extreme halophiles typically require NaCl concentrations exceeding 1.5 M for optimal development. They are ubiquitous and are widely distributed in habitats classified as salt lakes, hypersaline soils or sediments, salterns, and salt mines (Oren, 2002). To survive under osmotic stress, these organisms have evolved unique biochemical and physiological mechanisms. Their primary adaptation is the 'salt-in' strategy, maintaining high intracellular K^+ and Cl^- levels. They also possess specialized membrane lipids, an S-layer, and numerous acidic proteins that require high salt concentrations to maintain their native structure (Galinski, 1993; Oren, 1999). These adaptations make them a valuable source of biologically active compounds. Among the most significant compounds produced by halophiles are salt-resistant extracellular enzymes, pigments, and biosurfactants. These biomolecules have broad industrial and environmental applications due to their stability under extreme conditions (Antón et al., 2002; Mnif et al., 2009; Subramanian & Gurunathan, 2020).

Halophilic archaea are known for their ability to produce extracellular hydrolytic enzymes, such as amylases, proteases, and lipases (Qiu et al., 2021; Moopantakath et al., 2022; Song et al., 2023). Due to their remarkable stability and optimal activity under extreme environmental conditions, including high salinity and temperature, these exoenzymes are of both theoretical and practical interest. Their unique properties grant them significant value in industrial applications, such as detergent formulation, biofuel production, and pharmaceutical synthesis (Kasirajan et al., 2020; Schreck et al., 2021). For example, halophilic proteases have been employed in food processing and leather industries due to their catalytic efficiency at high salt concentrations (Antón et al., 2002).

Halophiles frequently produce carotenoid pigments, with bacterioruberin (C50 carotenoid) being the most characteristic in

extremely halophilic archaea. These microorganisms form colonies in shades of pink, orange, or red due to the high accumulation of bacterioruberin and related pigments. Such compounds absorb solar radiation and also protect the cells from photooxidative damage, shielding them against oxidative stress caused by UV exposure and high salinity. Beyond their protective role, C50 carotenoids exhibit strong antioxidant, antimicrobial, and anticancer properties, highlighting their relevance in pharmaceutical and cosmetic applications. Their stability and intense coloration enhance their potential as natural colorants (Antón et al., 2002; Subramanian & Gurunathan, 2020; Bouhamed et al., 2024).

The present study aimed to isolate several new halophilic microorganisms capable of producing biomolecules, including extracellular enzymes and carotenoid pigments, from different saline habitats, with a key role in their survival under extreme conditions.

MATERIALS AND METHODS

The samples used in this study were collected from two salt wells in Curmătura (Latitude: 45°09′25.307″ N, Longitude: 26°09′06.006″ E, Altitude: 213 m) and Băicoi (Latitude: 45°2′34.553″ N, Longitude: 25°53′18.667″ E, Altitude: 265 m), both located in Prahova County, Romania**.** We gathered and examined mud from Curmătura, and soil from the Băicoi salt well.

Isolation and characterization of biomolecule-producing halophiles

For the isolation and cultivation of halophilic microorganisms, we used JCM 168 medium (Rasooli et al., 2016), which contained the following components (g/L): NaCl (200), KCl (2), $MgSO_4 \cdot 7H_2O$ (20), $FeCl_2 \cdot 4H_2O$ (0.036), $MnCl_2 \cdot 4H_2O$ (0.00036), casamino acids (5), yeast extract (5), sodium glutamate (1), trisodium citrate (3), and agar (20)**,** with a final pH of 7.0. Decimal dilutions (10^{-1}-10^{-3}) of the samples were prepared in 3.4 M NaCl solution and inoculated by the pour-plate method (Sanders, 2012) in JCM 168 agar. The Petri plates were incubated at 37 °C up to 20 days. The number of viable microorganisms in samples was expressed as colony-forming units (CFU/g). The newly isolated strains were purified by multiple subculturing on JCM 168 agar medium and stored in glycerol at -80°C for long-term preservation. The new four isolates were further characterized by their phenotypic characteristics, including colony color, growth temperature, salt tolerance (0–5 M NaCl), production of extracellular hydrolytic enzymes, and molecular characteristics, specifically the 16S rRNA gene. Genomic DNA was extracted from isolates using the Pure Link genomic DNA kit (Invitrogen, Carlsbad, CA, USA). The 16S rRNA gene was amplified via polymerase chain reaction (PCR) using extracted genomic DNA, universal primers 27f/1492r (Marchesi et al., 1998) and 20f/1492r (Orphan et al., 2001), alongside GoTaq G2 Hot Start Polymerase (Promega, Madison, WI, USA), as previously described (Stancu, 2020; 2023; 2025). The thermal cycling conditions were carried out on an Eppendorf Mastercycler Pro S thermocycler (Hamburg, Germany) and included an initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were visualized onto 1.5% (w/v) agarose gels stained with SYBR Safe (Invitrogen) (Stancu, 2020; 2023; 2025).

The four isolates were screened to produce extracellular hydrolases, including lipase, protease, amylase, cellulase, xylanase, and pectinase, using the plate assay method (Rohban et al., 2008). The composition of the JCM 168 medium was modified by removing casamino acids, and reducing yeast extract to 1 g/L, and supplementing with 1 g/L of specific substrates such as Tween-80, casein, starch, carboxymethylcellulose, xylan, and pectin. Cultures were spot-inoculated onto the surface of the medium and incubated at 37°C for 10 to 20 days. Enzymatic activity was determined based on halo formation around the cultures.

The four isolates were further screened to produce carotenoid pigments. After reaching the stationary phase, the cultures were centrifuged, and the biomass was resuspended in acetone to extract pigments. Carotenoid extracts analysis was conducted using UV-visible spectroscopy, with absorbance measured from 200 to 800 nm (SPECORD 200

UV-Vis spectrophotometer, Analytik Jena, Jena, Germany). Total carotenoid content was determined at 494 nm using an absorption coefficient of 2500 (Hiyama et al., 1969). High-Performance Thin-Layer Chromatography (HPTLC) analysis was further performed using a CAMAG system, with samples applied to silica gel plates and developed in a chloroform-methanol (90:10, v/v) mobile phase (Stancu, 2020). The TLC plates were scanned under UV light (366 nm) for pigments examination. Antimicrobial activity of carotenoid extracts was evaluated using the agar diffusion techniques (Gómez-Villegas et al., 2020). The reference strains tested were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. Tryptic Soy Agar (TSA) plates were inoculated with 0.5 McFarland standard suspension of each pathogen. After drying, pigment extracts (10 µL), were spotted onto the medium. Acetone toxicity was ruled out before testing. Gentamicin (10 µg) and Fluconazole (25 µg) were used as positive controls. Petri Plates were incubated at 37°C for 24 hours.

RESULTS AND DISCUSSIONS

Romania has over 300 salt deposits, located either inside or outside the Carpathian Arc, formed through the evaporation of seawater and later shaped by tectonic processes, leading to the formation of salt massifs, saline springs, and hypersaline lakes (Cavruc & Chiricescu, 2006). Due to their high purity and accessibility, these deposits have been exploited over time for industrial purposes, balneotherapy, and human consumption. To make use of these resources, people have built salt wells, using their water for various food preservation, such as pickles and cheeses. Similar saline wells have been identified in Prahova County, specifically on the outskirts of Curmătura village and Băicoi town. These two saline wells, characterized by distinct physicochemical properties, including salinity exceeding 280 g/L, served as sources for the halophilic microorganisms' isolation (Figure 1.a).

Isolation and characterization of biomolecule-producing halophiles

From Curmătura mud and Băicoi soil we identified a microbial density of 10^4 CFU/g for both samples. Among the 27 isolates obtained in pure culture, four colonies with distinct morphological traits (Table 1) were chosen for further analysis. These colonies were designated as NC18, NC21, and NC28, all isolated from Curmătura mud, while SB1 was obtained from the Băicoi soil sample. Sensitivity tests to chloramphenicol (a broad-spectrum antibiotic) and sodium deoxycholate (a bile salt detergent), showed that all strains were resistant to chloramphenicol. This response differentiates them from typical bacteria and is consistent with established archaeal traits. Unlike bacteria, haloarchaea often exhibit antibiotic resistance patterns, including ampicillin, tetracycline, erythromycin, and chloramphenicol which generally target peptidoglycan synthesis and/or bacterial ribosomal structures (Thombre et al., 2016). Our results showed that all isolates were sensitive to sodium deoxycholate (Table 1), consistent with literature indicating that most non-coccoid halophilic archaea are more susceptible to bile salts than many bacteria. This sensitivity is likely due to structural variations in the membrane and S-layer, which differ among archaeal genera and species (Elevi & Oren, 2008). The four isolates exhibit remarkable salt tolerance, thriving in 1-5 M NaCl. Thus, NC18 grew from 1 up to 4 M salt, while NC21 and NC28 tolerated 1-5 M, and SB1 grew only in 2-5 M NaCl. Such high salt requirements are characteristic of halophilic Archaea. Extreme halophiles typically require over 1.5 M NaCl to develop, with optimal proliferation often at 3.4-5.2 M NaCl (Oren, 2002). Their growth temperature of 37 °C is within the mesophilic range common for many haloarchaeal species which generally grow well at 30-45 °C, with some able to exceed 50°C (Bowers et al, 2011).

Focusing on the amplification of the 16S rRNA gene, PCR-based molecular techniques were employed for the genetic characterization of the four isolates. All strains were successfully amplified using archaeal-specific primers (20f/1492r), confirming their classification within the Archaea domain, with the

characteristic 1472 bp amplicon obtained through PCR (Table 1, Figure 1c). No amplification was observed with bacterial-specific primers (27f/1492r) in any strain except NC28, suggesting the general absence of bacterial DNA. However, the amplification observed in NC28 could be due to horizontal gene transfer, which may have introduced the 16S rRNA gene of bacteria into the archaeal genome.

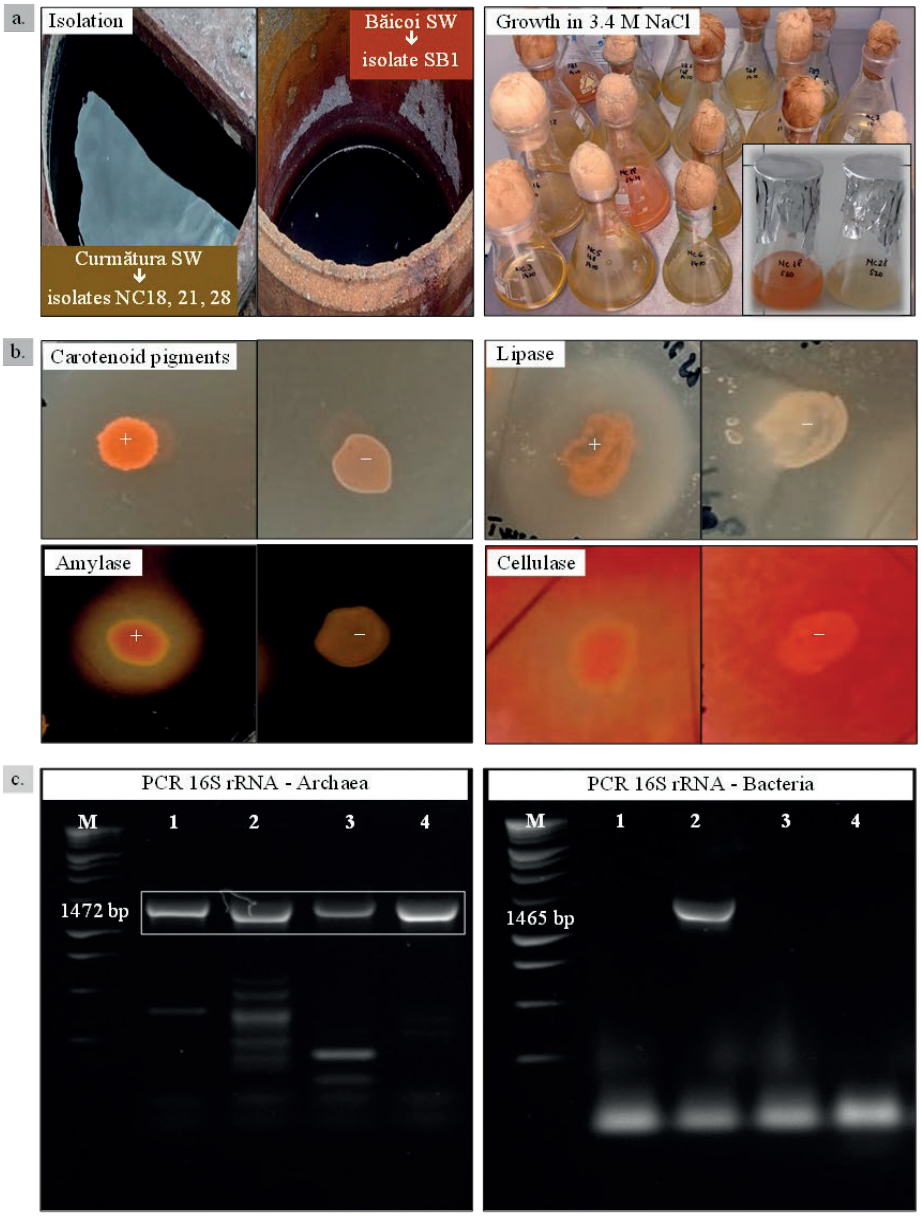

Figure 1. Isolation of halophiles from saline samples and their characterization: a. Curmătura and Băicoi salt well (SW) isolates NC18 (1), NC28 (2), NC21 (3), SB1 (4) grown in JCM168 medium with 3.4 M NaCl; b. isolate characterization, carotenoids, lipase, amylase, and cellulase, positive reaction (+), negative reaction (–); c. PCR of 16S rRNA gene using gDNA extracted from strains NC18 (1), NC28 (2), NC21 (3), SB1 (4), 1 kb DNA ladder (M)

Table 1. Characterization of halophiles isolated from saline samples

Characteristics	Strain			
	NC18	**NC21**	**NC28**	**SB1**
Isolation source	Curmătura mud	Curmătura mud	Curmătura mud	Băicoi soil
Growth on				
JCM168 agar	+	+	+	+
Chloramphenicol	+	+	+	+
Sodium deoxycholate	–	–	–	–
Color of colonies	Red	orange	orange	orange
Temperature growth (°C)	37	37	37	37
Salt tolerance capacity	1–4 M	1–5 M	1–5 M	2–5 M
PCR 16S rRNA gene using primers for				
Archaea (1472 bp)	+	+	+	+
Bacteria (1465 bp)	–	–	–	–
Extracellular hydrolase production				
Lipase (Tween80)	–	+	+	+
Protease (casein)	–	–	–	–
Amylase (starch)	+	+	+	–
Cellulase (carboxymethylcellulose)	–	+	+	+
Xylanase (xylan)	–	–	–	+
Pectinase (pectin)	–	–	–	–
Carotenoid pigments production	+	+	+	+
HPTLC (R_f)	0.07–0.61	0.19–0.69	0.09–0.23	0.22–0.26
Antimicrobial activity				
E. coli ATCC25922	+	+	+	–
P. aeruginosa ATCC 15442	–	–	–	–
S. aureus ATCC25923	–	–	–	–
C. albicans ATCC 10231	+	+	+	–

Positive reaction (+), negative reaction (–).

As confirmed by genetic and physiological characterization, the isolates belonging to the haloarchaea were further examined for their biotechnological potential by testing their ability to produce extracellular enzymes and carotenoid pigments.

Extremophilic enzymes, particularly extracellular ones, have a lot of promise for industrial applications due to their stability in high-salt environments and their ability to function in unconventional conditions (Moopantakath et al., 2022). In this study, the synthesis of six extracellular hydrolytic enzymes by haloarchaea isolates was assayed qualitatively (Table 1, Figure 1b). Several strains exhibited combined enzymatic activities, with NC21 and NC28 isolates capable of synthesizing amylase, lipase, and cellulase, while SB1 isolate synthesized lipase, cellulase, and xylanase. In contrast, NC18 displayed only amylase activity. No strains were capable of producing protease or pectinase. The findings are consistent with existing literature that indicates haloarchaea commonly produce amylase and lipase, which have applications in starch hydrolysis, biofuel production, and biocatalysis (Menasria et al., 2018). Extracellular enzymes, such as cellulase and xylanase, are less commonly found in halophilic Archaea (Zhang et al., 2011). Thus, their presence in certain strains is noteworthy. Our findings highlight the metabolic diversity of the haloarchaea isolates, their capability to produce multiple extracellular enzymes, and their consequent potential for diverse biotechnological applications.

One of the most prominent and conserved phenotypes observed in haloarchaea in their respective natural environments is the ability to produce carotenoids. UV-Vis spectra (in the domain 200-800 nm) of the carotenoid pigments extracted from the strains isolated in this study showed differences in their composition (Figure 2b). The absorbance spectrum of the red pigment extract from the NC18 showed a profile with maxima at 440, 500, and 530 nm. The peaks recorded at 500 and 536 nm are comparable to those documented for bacterioruberin (495 and 530 nm) (Bouhamed et al., 2024), the predominant C_{50} carotenoid in haloarchaea, indicating the potential presence of at least one variant of bacterioruberin.

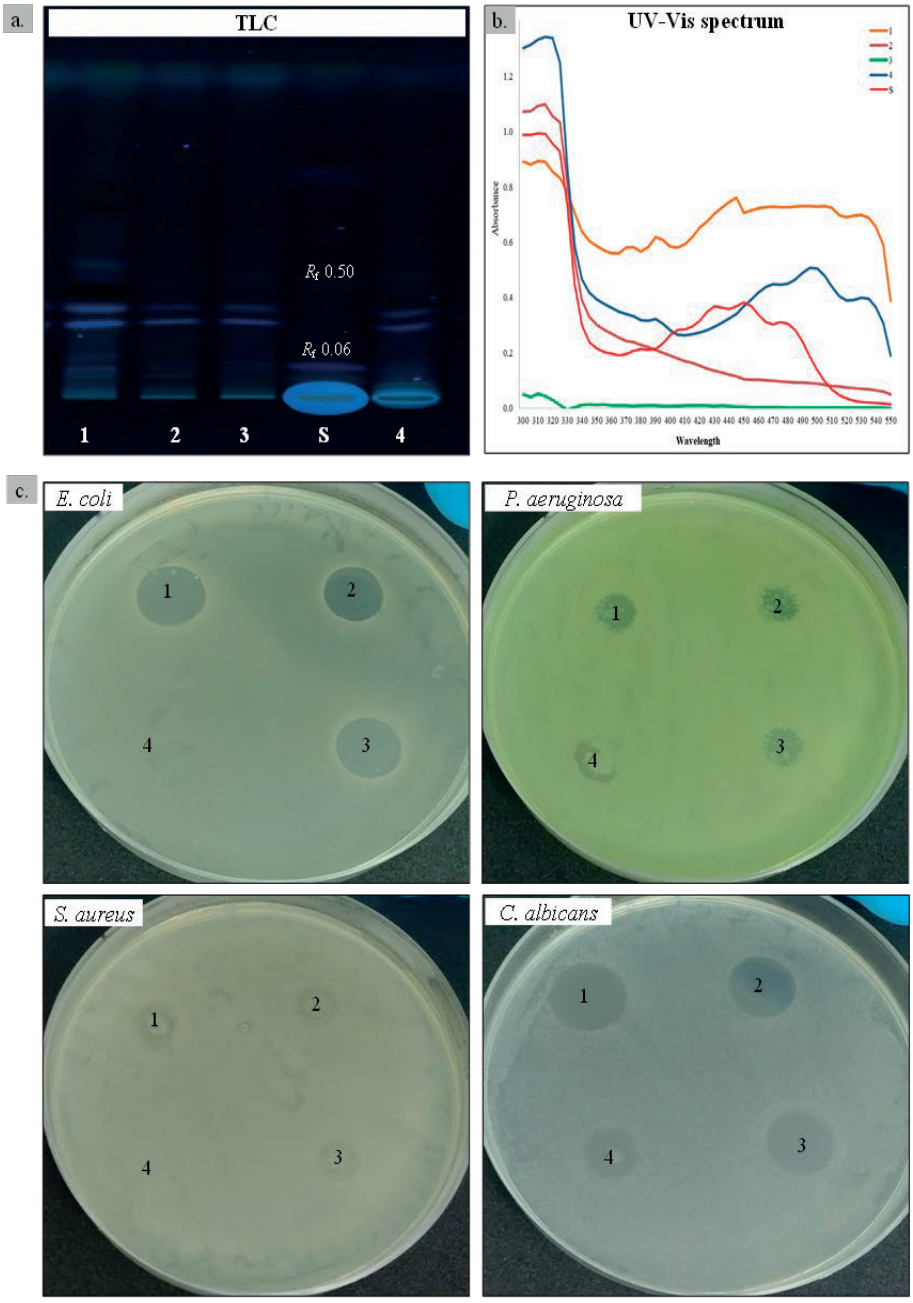

Figure 2. Carotenoid pigments production by halophiles: a. TLC of carotenoids extracted from strains NC18 (1), NC21 (2), NC28 (3), SB1 (4) grown in JCM168 medium with 3.4 M NaCl; retardation factor (Rf), carotenoid standards (S); b. UV-Vis spectrum of carotenoids (300-550 nm); c. antimicrobial activity of carotenoids extracted from strains NC18 (1), NC21 (2), NC28 (3), SB1 (4) on references pathogenic microorganisms

Given its known roles in photoprotection, membrane stability, and oxidative stress resistance, bacterioruberin presence may contribute to the survival and adaptation of these archaea in extreme environments. Additionally, its industrial potential in

biotechnology and pharmaceuticals further underscores the relevance of these results. The orange pigment extract from the SB1 also displayed three distinct absorption peaks at 470, 495, and 530 nm. This aligns with the findings of Kesbiç et al. (2023), who reported bacterioruberin absorption maxima at 467, 494, and 527 nm, values closely matching those observed in SB1. In contrast, the NC21 and NC28 did not exhibit peaks at these wavelengths (440-530 nm). However, all strains showed a peak at ~310 nm, likely corresponding to carotenoid precursors present in the cells, such as phytoene or phytofluene. Although no absorption peak in the visible range was identified for extracts from NC21 and NC28, HPTLC analysis revealed several visible bands in all the halophiles extracts, confirming the presence of carotenoid pigments with distinct R_f values ranging from 0.07 and 0.69 (Table 1, Figure 2a). The superior sensitivity of TLC, which may separate trace pigments undetectable by UV-Vis spectroscopy, may be responsible for this discrepancy. For better understanding of carotenoid production, the total carotenoid concentrations were further determined. NC18 and SB1 were the highest carotenoid producers, yielding 585.2 µg/mL and 406.2 µg/mL, respectively, while NC21 (84.2 µg/mL) and NC28 (52.0 µg/mL) produced significantly lower amounts, consistent with our previous results.

The antimicrobial activity of the carotenoid pigment extracts was assessed against the pathogenic reference strains *E. coli*, *S. aureus*, *P. aeruginosa,* and *C. albicans*. Our results revealed that the carotenoid extracts from NC18, NC21, and NC28 exhibited inhibitory effects against *E. coli* and *C. albicans*, but not against *P. aeruginosa* or *S. aureus* (Figure 2c). Interestingly, the SB1 pigment extract had no antimicrobial activity against any of the pathogens tested, despite the high carotenoid concentration. According to our results, stability, bioavailability, and composition of pigments are important factors in antibacterial activity. These results indicate that pigment stability, bioavailability, and composition are key to antimicrobial efficacy. The specific chemical structures of the pigments from NC18, NC21, and NC28 might interact more effectively with the membranes or metabolic pathways of *E. coli* and *C. albicans,* while the inherent resistance mechanisms in *P. aeruginosa* and *S. aureus* could inhibit these interactions. Moreover, the lack of activity in the SB1 extract shows that a high carotenoid concentration alone is not sufficient for antimicrobial action.

This underscores the necessity for an in-depth investigation of haloarchaeal carotenoids, particularly their structural and functional properties, to explore their potential in biotechnological applications. Halophilic microorganisms present sustainable solutions to industrial and environmental challenges, while their distinctive metabolic pathways offer valuable insights into extremophile adaptations, facilitating the development of innovative biotechnological applications (Antón et al., 2002; Subramanian & Gurunathan, 2020; Bouhamed et al., 2024).

CONCLUSIONS

Based on the phenotypic and molecular characteristics, the four newly isolated strains, designed as NC18, NC21, NC28, and SB1, were included in the Archaea domain. Our results highlight the remarkable ability of the halophilic isolates to thrive in extreme environments, making them promising candidates for industrial processes that require stability under harsh conditions. Their capability to synthesize biomolecules, including extracellular hydrolytic enzymes and carotenoid pigments under extreme environmental conditions (NaCl 3.4 M, pH 7.0, and 37 °C) minimizes the dependency on synthetic chemicals, thereby supporting the principles of green technology.

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