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Optimization of Growth Media for Halophilic Bacteria Isolate from Salt Pond: A Kinetic Analysis Across Different Agar Substrates

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ABSTRACT

Halophilic bacteria are extremophilic microorganisms with significant ecological and industrial importance, particularly for biotechnological applications such as enzyme production, bioremediation, and saline wastewater treatment. This study aimed to optimize culture media to enhance halophilic bacterial growth performance by evaluating four agar-based substrates with different nutrient compositions: Nutrient Agar Modified (NAM), Mannitol Salt Agar (MSA), Total Plate Agar Modified (TPAM), and Casein Medium Agar (CMA). The bacterial isolate, obtained from hypersaline salt ponds (40% salinity) in East Java, Indonesia, was cultured for seven days under controlled laboratory conditions. Growth was quantified daily using colony-forming unit (CFU) counts, and kinetic parameters including maximum abundance (Y_max), specific growth rate (μ_max), generation time (g), and cumulative growth momentum (AUC-μ) were analyzed. The results revealed that CMA produced the highest growth rate (μ_max = 0.27 day^{-1}), the shortest generation time (g = 2.56 days), and the greatest overall growth momentum (AUC-μ = 0.436), indicating efficient nitrogen assimilation and rapid cell division under hypersaline stress. In contrast, MSA exhibited the slowest growth (μ_max = 0.06 day^{-1}) and longest generation time (g = 11.9 days). These findings confirm that medium composition strongly influences halophilic bacterial kinetics through nutrient assimilation and osmotic adaptation. Casein-based formulations such as CMA may therefore serve as a baseline medium for halophilic enzyme production and saline bioprocess development.

INTRODUCTION

Halophilic and halotolerant bacteria represent a remarkable group of microorganisms capable of thriving under extreme salinity where most life forms cannot

survive. Their ecological and physiological resilience has drawn considerable scientific attention for both fundamental and applied purposes. In natural hypersaline systems such as salt ponds, brine pools, and saline lakes, these microorganisms sustain vital ecological processes through osmotic regulation, compatible solute accumulation, and enzymatic adaptation **(Mallik *et al.*, 2024; Martínez-Espinosa *et al.*, 2024)**. Recent advances in metagenomics and microbial cultivation have uncovered diverse halophilic taxa with potential for biotechnological applications, including bioremediation of saline wastewater, enzyme production, and bioprocessing in high-salt environments **(Yavari-Bafghi & Amoozegar, 2025)**.

Despite extensive exploration of halophilic diversity, quantitative understanding of their growth kinetics under different nutrient regimes remains limited **(Oren, 2024; Paulo *et al*, 2024; Kurniawan *et al.*, 2025; Mas'ud *et al.*, 2025)**. Most previous studies have focused on taxonomic characterization or qualitative growth responses rather than precise kinetic behavior across varying substrate compositions. Furthermore, while it is well established that halophilic microorganisms exhibit slow growth relative to non-halophiles due to osmotic constraints and energetic costs, the influence of nutrient type particularly the contrast between nitrogen-rich and carbohydrate-based media on their kinetic parameters has not been systematically evaluated **(Kurniawan *et al.*, 2024a; Liu *et al.*, 2025; Rezaei *et al.*, 2025)**. Consequently, the relationships among nutrient composition, osmoadaptation energy demand, and cell proliferation dynamics remain poorly defined.

Conventional approaches to microbial growth studies typically emphasize single-point measurements such as maximum cell yield or peak optical density, which overlook the dynamic temporal patterns that characterize halophilic metabolism **(Eskelin & Oksanen, 2021; Gutiérrez-Preciado *et al.*, 2024)**. Advanced analyses integrating both instantaneous growth rate and cumulative growth momentum are rarely applied to halophilic systems. This gap limits the capacity to assess not only how fast these bacteria grow but also how consistently they sustain growth across time under hypersaline stress. Addressing this knowledge gap is essential for optimizing cultivation strategies and designing high-performance media for halophilic biotechnological applications.

Understanding the growth kinetics of halophilic bacteria under various nutrient and salinity conditions is also fundamental for scaling up bioprocesses into industrial bioreactors. Kinetic parameters such as maximum specific growth rate, generation time, and cumulative growth momentum directly influence reactor design, nutrient feeding strategies, and oxygen transfer efficiency. Insights from solid-medium kinetics can thus be translated into predictive models for liquid-culture performance, supporting the optimization of large-scale enzyme induction and saline wastewater biotreatment systems. This integration of laboratory kinetics with bioreactor modeling forms a critical step toward the commercialization of halophilic-based biotechnologies.

The present study aimed to characterize and compare the growth kinetics of halophilic bacterial isolates cultivated on four distinct agar-based media representing different nutrient compositions: protein-based, carbohydrate-based, and two general-purpose modified formulations. By analyzing the temporal distribution of specific growth rates and integrating the cumulative momentum of growth over incubation time, this research provides a comprehensive evaluation of how medium composition influences growth dynamics under saline conditions. In addition to quantifying growth performance, the study offers insights into the metabolic and adaptive strategies underlying halophilic resilience. The findings are expected to contribute both to fundamental microbial physiology and to the applied development of efficient halophilic bioprocesses for saline wastewater treatment and enzyme production.

MATERIALS AND METHODS

Experimental design

The halophilic bacterial isolate used in this study was obtained from the Laboratory of Fishery Product Safety, Universitas Brawijaya. The isolate had been successfully recovered by **Al Zamzami *et al.* (2025a)** from hypersaline salt ponds located in East Java, Indonesia, characterized by salinity levels reaching 40%. Salinity was precisely quantified using a Smart Salt Detector (SD-B01071), ensuring reliable measurement under field conditions. The selection of this isolate was based on its demonstrated tolerance to extreme salt concentrations, which makes it a suitable model organism for growth kinetics studies under hypersaline stress. The culture was maintained under controlled laboratory conditions prior to experimentation, and inocula were prepared following standard microbiological techniques to ensure reproducibility across treatments.

Growth media

The halophilic bacterial isolate was cultured under controlled laboratory conditions to evaluate its growth dynamics on four agar-based media (Table 1): Nutrient Agar Modified (NAM), Mannitol Salt Agar (MSA), Total Plate Agar Modified (TPAM), and Casein Medium Agar (CMA). Each medium was prepared according to standard microbiological protocols, sterilized by autoclaving at 121°C for 15 minutes, and poured into sterile Petri dishes. The inoculation was performed aseptically, and cultures were incubated at 37°C for seven days. Colony counts were measured daily using the colony-forming unit (CFU) method and expressed as CFU/mL to monitor growth over time. All treatments were conducted in triplicate biological replicates to ensure statistical reliability and reproducibility of kinetic parameters.

Table 1. Selected agar compositions

Medium Composition	NA Mod	MSA	TPA Mod	CMA
Lab-lemco powder	1 g/L	1 g/L		
Yeast extract	2 g/L		2,5 g/L	5 g/L
Peptone	5 g/L	10 g/L	5 g/L	7,5 g/L
Sodium chloride	75 g/L	75 g/L	75 g/L	75 g/l
Agar	15 g/L	15 g/L	15 g/L	15 g/L
Mannitol		10 g/L		
Phenol red		0,025 g/L		
$MgCl_2 . 6H_2O$				2 g/L
KCl				2 g/L
$CaCl_2$				0,2 g/L

Growth kinetics analysis

Growth performance was quantified using classical microbial growth kinetics. Daily CFU data were plotted against incubation time (days) to generate growth curves for each medium. From these curves, five key parameters were determined:

a) Maximum abundance (Ymax): the highest CFU mL^{-1} observed during incubation.
b) Lag time (λ): the period before the onset of exponential growth.
c) Exponential phase (t start – t end): the time window of rapid cell division.
d) Maximum specific growth rate (μmax): derived from the slope of the exponential growth curve.
e) Generation time (g): the time required for the population to double, calculated as:

$$g = \frac{\ln 2}{\mu_{max}} \quad (1)$$

The value of μmax (day^{-1}) was determined by fitting the exponential portion of the growth curve using linear regression:

$$\mu_{max} = \frac{\ln N_t - \ln N_0}{t} \quad (2)$$

Where, N_t is the cell concentration at time ttt during the exponential phase, and N_0 is the initial cell concentration at the start of the exponential phase.

Cumulative Growth Momentum (AUC-μ)

To evaluate overall growth performance, the cumulative area under the μ–time curve (AUC-μ) was calculated as a dimensionless parameter representing total growth momentum over the 7-day incubation period:

$$\mathrm{AUC} - \mu = \sum_{i=1}^{n-1} \mu_i \, \Delta t \quad (3)$$

Where, μ_i represents the specific growth rate between two successive sampling times and Δt = 1 day.

The resulting AUC-μ values were used to compare cumulative growth performance among media, with higher values indicating greater total growth efficiency.

RESULTS

Growth dynamics

Across media, growth diverged sharply. MSA showed the earliest adaptation (day 3) and the highest biomass, stabilizing at 19.7 CFU/mL by day 6, making it the most effective substrate. NAM exhibited a long lag (no growth through day 3) and a low plateau (4.0 CFU/mL by day 5), indicating poor nutrient support. TPAM behaved similarly to NAM with late, minimal growth (up to 4.0 CFU/mL by day 7). CMA initiated earlier (9.7 CFU/mL on day 3) and rose steadily to 15.0 CFU/mL by day 6, suggesting casein supplies a more accessible nitrogen source that accelerates the exponential phase, though final yield trails MSA.

Table 2. Abundance of halophilic bacteria across different agar media

Medium Type	Bacteria Abundance (CFU mL^{-1})						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
NAM	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.33 ± 0.6	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
MSA	0.0 ± 0.0	0.0 ± 0.0	16.67 ± 0.6	17.67 ± 0.6	18.67 ± 0.6	19.67 ± 0.6	19.67 ± 0.6
TPAM	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.67 ± 0.6	3.0 ± 0.0	3.67 ± 0.6	4.0 ± 0.0
CMA	0.0 ± 0.0	0.0 ± 0.0	9.67 ± 0.6	12.67 ± 1.5	14.67 ± 0.6	15.0 ± 0.0	15.0 ± 0.0

Growth kinetics of halophilic bacteria on different agar media

The kinetic analysis revealed distinct growth performance of halophilic bacterial isolates across the four tested agar media (Table 3 & Fig. 1). The highest maximum growth rate (μ max) was recorded on Casein Medium Agar (CMA) at 0.27 day^{-1}, followed by Total Plate Agar Modified (TPAM) (0.20 day^{-1}) and Nutrient Agar Modified (NAM) (0.18 day^{-1}). The lowest μ max value was observed on Mannitol Salt Agar (MSA) with 0.06 day^{-1}. The generation time (g) varied inversely with μ max, ranging from 2.56 days in CMA to 11.90 days in MSA.

Table 3. Kinetic parameters of halophilic bacteria across different agar media

Medium Type	Y max (CFU mL^{-1})	λ (Day)	t start (Day)	t end (Day)	μ max (Day^{-1})	g (Day)
NAM	4.0	3	4	5	0.18	3.80
MSA	**19.7**	2	3	5	0.06	**11.90**
TPAM	4.0	3	4	6	0.20	3.45
CMA	15.0	2	3	5	**0.27**	2.56

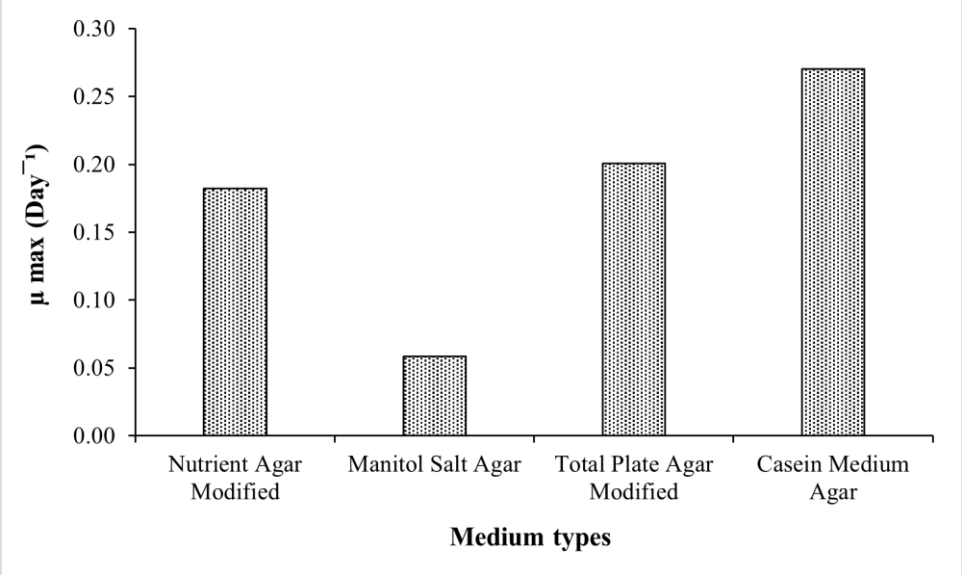

Fig. 1. Maximum growth rate distribution of halophilic bacteria

The maximum cell abundance (Y max) also varied across media, with the highest value of 19.7 CFU mL^{-1} observed in MSA, followed by CMA at 15.0 CFU mL^{-1}. NAM and TPAM both reached a Y max of 4.0 CFU mL^{-1}. The lag phase (λ) was the shortest in MSA and CMA (2 days), while NAM and TPAM showed a longer lag period (3 days). The exponential growth phase typically began between days 3–4 and ended between days 5–6, indicating a consistent initiation of rapid cell multiplication across all tested media.

Based on the μ max distribution (Fig. 1), CMA demonstrated the steepest increase in maximum growth rate, whereas MSA exhibited a slower and delayed response. NAM and TPAM presented intermediate kinetic behavior with moderate acceleration during the exponential phase. These results highlight measurable differences in bacterial growth kinetics among the four media, with CMA showing the highest μ_max and shortest generation time, reflecting a stronger growth momentum under hypersaline culture conditions.

The temporal distribution of specific growth rates (μ day^{-1}) across the 7-day incubation intervals revealed dynamic variations among the four tested media (Table 4 & Fig. 2). No measurable growth was detected in any medium during the first two intervals (Days 1–3), confirming an initial lag phase across all treatments. Growth acceleration

began between days 3–4, particularly in Casein Medium Agar (CMA) with μ = 0.269 day^{-1} and Mannitol Salt Agar (MSA) with μ = 0.058 day^{-1}.

During the following interval (Day 4–5), specific growth rates increased in all media. Nutrient Agar Modified (NAM) recorded μ = 0.192 day^{-1}, Total Plate Agar Modified (TPAM) reached 0.105 day^{-1}, and CMA maintained 0.146 day^{-1}. MSA showed a secondary minor increase with μ = 0.055 day^{-1}. By Day 5–6, TPAM achieved its peak μ value of 0.21 day^{-1}, while CMA decreased to 0.02 day^{-1} and MSA maintained a moderate rate of 0.052 day^{-1}. No further increase in μ was observed beyond Day 6 in any treatment, indicating the cessation of exponential growth.

The cumulative area under the curve (Σμ or AUC–μ), representing the overall growth momentum, indicated a distinct ranking order: CMA > TPAM > NAM > MSA, with corresponding Σμ values of 0.436, 0.393, 0.192, and 0.165. Casein Medium Agar exhibited the highest integrated growth momentum, whereas Mannitol Salt Agar demonstrated the lowest. The temporal growth profile (Fig. 4) showed distinct peak intervals among the tested media. CMA reached its maximum μ during day 3–4, TPAM and NAM attained their peaks between days 4–6, while MSA maintained consistently low μ values throughout the incubation period.

Table 4. Kinetic parameters of halophilic bacteria across different agar media

Interval	NAM	MSA	TPA	CMA
Day 1–2	0	0	0	0
Day 2–3	0	0	0	0
Day 3–4	0	**0.058**	0	**0.269**
Day 4–5	**0.192**	**0.055**	**0.105**	0.146
Day 5–6	0	**0.052**	**0.21**	0.02
Day 6–7	0	0	0	0
Σ μ (AUC-μ)	**0.192**	**0.165**	**0.393**	**0.436**
Rank (Growth Momentum)	3^{d}	4th	2nd	**1st**

*The parameter Σμ (AUC-μ) represents the cumulative growth momentum, indicating the integrated total of specific growth rates over the seven-day incubation period.

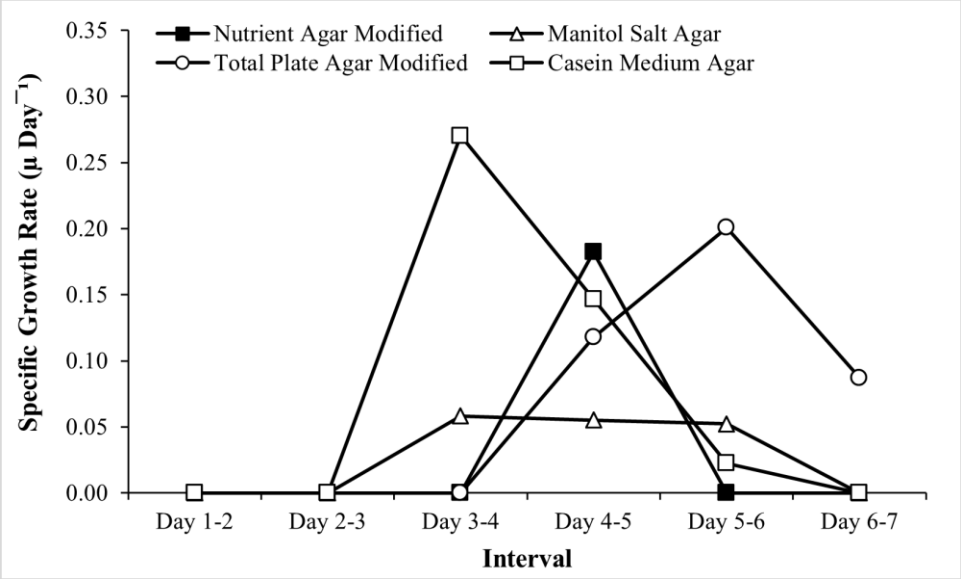

Fig. 2. Maximum growth rate distribution of halophilic bacteria

DISCUSSION

Influence of medium composition on growth performance

The overall growth behavior of the halophilic bacterial isolates demonstrated clear variation among the tested media, emphasizing the importance of nutrient form and availability under hypersaline conditions. The casein-based medium provided the most favorable environment for bacterial proliferation **(Nichols *et al.*, 2008; Huang *et al.*, 2025)**, as indicated by its higher rate of growth and shorter generation period compared with the other formulations. This result suggests that proteinaceous substrates are more efficiently assimilated than carbohydrate-based ones in saline stress environments.

The nutrient composition of the casein medium likely supplied readily available amino acids and peptides, promoting faster biosynthetic activity and cellular replication **(Kurniawan *et al.*, 2024b; Hao *et al.*, 2025; Moita *et al.*, 2025)**. In contrast, the mannitol salt medium, which is rich in carbohydrates, may have caused osmotic stress and reduced energy conversion efficiency. Such differences are consistent with the broader understanding that the maximum rate of bacterial growth is controlled by the rate of substrate assimilation and enzymatic activity under given environmental stresses **(Gonzalez & Aranda, 2023; Scott & Hwa, 2023)**.

Although the mannitol salt medium yielded a higher final abundance of bacterial cells than some other media, the growth rate remained slow. This decoupling between cell yield and metabolic turnover reflects physiological adaptation to osmotic stress, in which energy is diverted toward maintenance rather than replication. **Perwira *et al.* (2020)** and **Rajpurohit and Eiteman (2022)** noted that under nutrient limitation or high ionic pressure, microorganisms often exhibit high biomass yield aligned with reduced replication rates, supporting the pattern observed here.

Temporal growth momentum and interval dynamics

When viewed across time intervals, the casein medium again demonstrated the most consistent acceleration and sustained growth momentum. Followed by the total plate agar and nutrient agar formulations, the mannitol salt medium maintained the lowest momentum throughout incubation. The early and sharp activation of growth in the casein formulation indicates a rapid physiological response to nutrient availability, while the later peaks observed in total plate and nutrient agar suggest a slower adaptation phase before exponential proliferation.

The integrated measure of growth momentum across the entire incubation period offers a more comprehensive view of microbial performance than instantaneous growth rate alone. This cumulative parameter reflects both the intensity and the persistence of metabolic activity over time. As suggested by **Kobziar *et al.* (2024)**, evaluating microbial momentum through time-integrated analysis allows for a more accurate representation of adaptive dynamics in complex environments. The sustained performance observed in the casein and total plate media indicates that their nutrient compositions not only promote faster initiation of growth but also support metabolic stability through extended cultivation **(Hayek *et al.*, 2019; Yeboah *et al.*, 2023; Salamah *et al.*, 2024)**.

Halophilic adaptation and metabolic strategy

Halophilic bacteria adapt to high salinity through compatible solute accumulation or intracellular ion regulation, which stabilize enzymes and proteins. These mechanisms require high energy; thus, nitrogen-rich compounds from casein medium help reduce the metabolic cost of adaptation and enhance growth efficiency **(Al Zamzami *et al.*, 2025b)**. Both mechanisms are energetically demanding therefore, access to nitrogen-rich compounds may reduce the metabolic cost associated with de novo synthesis of amino acids and osmoprotectants **(Waditee-Sirisattha & Kageyama, 2022; Bilova *et al.*, 2024; Bilova *et al.*, 2025)**.

Protein-based substrates such as casein can also facilitate the activation of proteolytic and deaminase enzymes that are essential under saline stress. This is consistent with reports that halophilic microorganisms preferentially metabolize nitrogenous substrates when exposed to osmotic stress **(Zhang *et al.*, 2020; Ismail *et al.*, 2025)**. In this respect, studies highlighted that nitrogen sources enhance the production of compatible solutes, further improving osmotic balance and enzyme stability **(Liang *et al.*, 2020; Anggayasti *et al.*, 2025)**.

Carbohydrate-based media, such as the mannitol formulation, often display lower metabolic efficiency under saline stress because salt can interfere with carbohydrate transport systems and inhibit key glycolytic enzymes. Consequently, bacterial growth tends to proceed more slowly and with reduced energy yield. Similar patterns were described by **Robinson *et al.* (2005)**, who observed that halophilic archaea exhibited

rapid proliferation when provided with amino acid-rich environments compared to carbohydrate-based media.

Broader implications and future prospects

These findings collectively demonstrate that the efficiency of halophilic bacterial growth is closely tied to the biochemical nature of the substrate. Media enriched with complex nitrogen sources not only accelerate growth initiation but also maintain stable metabolic activity over time, making them particularly suitable for biotechnological applications such as saline wastewater bioremediation or enzyme production under hypersaline conditions.

The dual assessment of instantaneous growth rate and cumulative growth momentum provides a valuable framework for evaluating microbial performance in saline systems. Future studies should extend this approach by incorporating liquid-phase kinetics, metabolic flux analysis, and omics-based profiling to identify the genetic and enzymatic determinants underlying substrate preference. As highlighted by **Liu *et al.* (2021)** and **Yamagishi and Hatakeyama (2025)**, integrating empirical growth data with computational models can reveal how energy allocation and regulatory networks govern microbial adaptation in extreme environments. In addition, nitrogen-enriched formulations such as CMA could potentially enhance extracellular enzyme yield (protease, lipase) and stimulate halocin biosynthesis, offering new prospects for halophilic bioindustrial applications.

CONCLUSION

The growth kinetics of halophilic bacteria revealed a clear dependence on the biochemical composition of the growth medium. Among the four tested substrates, Casein Medium Agar (CMA) proved to be the most effective in promoting bacterial proliferation, showing the highest growth rate, the shortest generation period, and the greatest overall growth momentum. These parameters collectively demonstrate enhanced metabolic efficiency and rapid utilization of nitrogen-rich compounds under hypersaline stress conditions. Conversely, Mannitol Salt Agar (MSA) showed the slowest cell proliferation and the longest doubling time, indicating limited adaptation to carbohydrate-dominated environments with elevated ionic pressure. Total Plate Agar Modified (TPAM) and Nutrient Agar Modified (NAM) presented intermediate growth responses, with exponential phases generally occurring between the third and sixth day of incubation. The temporal kinetic pattern showed that CMA reached its fastest metabolic activation earlier in the incubation period, while TPAM exhibited a delayed but sustained acceleration. Protein-enriched media were more effective than carbohydrate-based formulations in supporting rapid and stable bacterial growth under saline stress. This highlights their potential value in biotechnological processes involving halophilic microorganisms, including saline wastewater bioremediation, enzyme production, and

bioreactor development. Future studies should verify these findings in liquid culture systems and integrate kinetic, molecular, and metabolic analyses to better elucidate substrate-specific adaptations and optimize halophilic cultivation for industrial and environmental use.

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