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Synergistic Interaction of *Spirulina Sp*. and Folic Acid- Producing Bacteria for Folate Production

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Abstract

Folate, an essential nutrient crucial for DNA synthesis, cell division, and fetal neural tube development, remains a global health concern due to deficiencies in certain populations. To address this issue, we investigated the synergistic interaction between *Spirulina*, a nutrient-rich microalga, and two strains of folic acid-producing bacteria, *Bacillus subtilis*-1 and *Bacillus subtilis*-2, to enhance folate production. *Spirulina* has high nutritional content, combined with the folate production capabilities of the selected bacteria, offered a promising opportunity for sustainable folate synthesis. In this study, *Spirulina* and the two strains of *Bacillus subtilis* were cultured separately to optimize growth conditions for each organism. Co-culture experiments were then conducted, combining *Spirulina* with *Bacillus subtilis*-1 and also *Bacillus subtilis*-2, to investigate their collective potential for folate production. The specific growth rates of both *Spirulina* and the bacteria were measured individually and in combination using spectrophotometric methods, and their dry weights were determined to assess biomass productivity. Folate quantification in the microalgal-bacterial cultures was performed using a spectrophotometric analysis based on the phosphate buffer extraction method. This method facilitated the measurement of folate content investigated the impact of the symbiotic relationship between *Spirulina* and bacteria, particularly in terms of enhancing vitamin B_{12} acquisition and its impact on folate synthesis. Our results revealed a synergistic enhancement

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in folate production at the exponential growth phase of *Bacillus subtilis*-1 and *Bacillus substilis-2* co-culture. The synergistic relationship between *Spirulina* and *Bacillus subtilis*-1, as well as *Bacillus subtilis*-2, is manifested in elevated folate synthesis, highlighting the significant impact of microbial cooperation on nutrient production. Additionally, we observed fluctuations in folate production at the stationary phase, highlighting the subtle equilibrium achieved through symbiotic interactions. These findings shed light on the potential of harnessing the synergistic potential of microorganisms for sustainable folate synthesis. This research advances co culturing methods to optimize folate production, tackling global folate deficiency challenges and fostering innovative, sustainable nutritional solutions.

Introduction

Spirulina, a filamentous cyanobacterium belonging to the genus Arthrospira,[1] exhibits remarkable versatility as a microorganism, boasting a rich history of human utilization.[2] Flourishing in diverse environments, including alkaline freshwater and saltwater, *Spirulina* has been utilizing as a dietary staple in various cultures worldwide.[3] Recent scientific investigations have cast a spotlight on *Spirulina* is potential as a source of folic acid.[4] This microorganism possesses inherent folate production capabilities.[5] Furthermore, when subjected to co-cultivation with folate-producing bacteria, *Spirulina* has demonstrated the capacity to enhance folic acid production, resulting in a synergistic effect on folate synthesis.[6] These discoveries unveil promising pathways for the creation of economical and environmentally sustainable methodologies for folic acid synthesis.

Folate, often known as vitamin B_9, is an essential nutrient that plays a variety of roles in important biological processes in the human body. Its significance extends from its pivotal involvement in DNA synthesis and cell division to its crucial contribution to fetal neural tube development,[7,8] However, despite its fundamental importance, the global challenge of folate deficiency persists, affecting specific populations and necessitating the exploration of innovative strategies for its amelioration.

Previous research has gone extensively into the multiple repercussions of folate deficiency and the many techniques used to address this worldwide health problem. Notably, low folate levels have been linked to an increased risk of various health problems. One of the most noticeable outcomes of folate deficiency is the incidence of neural tube abnormalities (NTDs) in growing fetuses.[9] The insufficiency of maternal folate intake during pregnancy has been linked to an increased risk of NTDs, severe malformations of the brain and spinal cord.[10] Additionally, diminished folate levels have been implicated in the development of various conditions, encompassing cardiovascular diseases, specific cancer types (such as colorectal, pancreatic, and cervical cancer), and neurodegenerative disorders like Alzheimers disease.[11] These investigations collectively underscore the critical nature of folate, especially during periods characterized by rapid growth, thereby emphasizing its indispensable significance in fetal development and overall health. In response to the persistent challenge posed by folate deficiency, we embarked on a research endeavor aimed at investigating an innovative approach to address this issue. Researchers are exploring the possibility of microbial fermentation to produce natural folate. Several biotechnological and chemical methods are used to make folic acid in high production scale, *Bacillus subtilis* has been modified to boost the synthesis of folate by co-culturing strategy. Microbial manufacturing is a sustainable and cost-effective technique to manufacture an appropriate blend of folate vitamins. Specifically, our study focused on examining the synergistic interaction between *Spirulina*, a microalga known for its rich nutritional content, and two folic acid-producing bacteria strains, *Bacillus subtilis*-1 and *Bacillus subtilis*-2, with the primary objective of augmenting folate production. The rationale behind this investigative pursuit stemmed from the remarkable nutritional profile exhibited by *Spirulina*, coupled with the inherent folate production capabilities exhibited by the chosen bacterial strains. Our methodological approach entailed the

separate cultivation of *Spirulina* and the two strains of *Bacillus subtilis*, allowing for the optimization of growth conditions tailored to each organisms' unique requirements. Subsequently, co-culture experiments were conducted, bringing together *Spirulina* with *Bacillus subtilis*-1 and *Bacillus subtilis*-2, respectively, to explore their combined potential for folate production. The outcomes showed the profound impact of microbial cooperation on nutrient production. The implications of this research venture extend beyond the laboratory, offering insights into the collaborative potential of microorganisms and holding significant promise for addressing global challenges related to folate deficiencies. The synergistic association between *Spirulina* and folic acid-producing *Bacillus subtilis* strains presents a promising approach for the development of functional foods enriched with folate, thus contributing to improved health outcomes. Moreover, the study's objective was to harness a microalgae-heterotrophic bacteria co-culture, specifically between *Spirulina* and *Bacillus subtilis*, to boost folate production, aiming for functional foods with enhanced nutritional value.Ultimately, these findings offer innovative, sustainable, and eco-friendly approaches to meet the ever-growing nutritional demands of our global populace.

Materials and Methods

The cyanobacteria and bacteria that showed the highest folate production capabilities were selected for co-culture experiments. The co-culture experiments were conducted using a two-step process. In the first step, the folic acid producing bacteria and Cyanobacteria Specifically *Spirulina* were cultured separately in a nutrient-rich medium. In the second step, the folic acid producing cyanobacteria were added to the bacterial culture at different time intervals (24 Hours). The co culture experiments were conducted under different environmental conditions, and the folate production was measured at different time points using standard microbiological techniques. The folate production was quantified using Phosphate Buffer Extraction Method.

Study Area

Bacterial strains from yogurt and *Spirulina*sp. from Usterzai, Kohat, KPK, Pakistan, were previously identified in a lab project. The experiment was conducted at the Laboratory of Molecular Ecology and Conservation, Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, KP, Pakistan.

Culture Cultivation for Consortia

In this study, the cultivation techniques.[12] were followed to establish large-scale and sustainable cultivation of microalgae. These techniques are crucial for ensuring optimal growth and productivity of microalgae cultures. They involve providing suitable growth conditions such as temperature, light intensity, humidity and pH in a growth chamber specially design for this experiment. The cultivation setup consisted of culture flasks, depending on the scale of the experiment. *Spirulina* sp. is cultured in BG-11 media, while bacterial strains for the study are maintained in MRS broth media.

Furthermore, the methods.[13] were adopted to establish microalgal-bacterial interactions in aquaculture systems. The microalgae-bacteria consortia were carefully prepared by inoculating the microalgae culture with the selected bacteria under controlled laboratory conditions like pH -10, temperature-22°C and light intensity 2400lux. This approach aimed to promote a synergistic relationship between microalgae and bacteria, resulting in increased folate production.

Specific Growth Rate Measurement of Folate-Producing Bacteria and *Spirulina* for Enhanced Folate-Production

To determine the specific growth rate of folic acid-producing bacteria[14] and *Spirulina sp*., a widely employed spectrophotometric method was utilized for folic acid-producing bacteria and for *Spirulina* sp.[15]

For folic acid-producing bacteria, the samples were analyzed at regular intervals (24 Hours), and their optical density (OD) was measured at a specific wavelength of 580nm using a spectrophotometer The OD measurement at 580nm is commonly used to estimate the cell concentration in bacterial cultures, as it correlates with the microbial biomass present in the sample. The increase in OD over time allowed us to determine the specific growth rate of the folic acid-producing bacteria accurately.

For *Spirulina* sp., the spectrophotometric method was adopted for OD measurements at 580nm.[15] This technique facilitated the calculation of *Spirulina*'s specific growth rate by analyzing OD value changes.

Dry Weight Analysis of Folate-Producing Bacteria and *Spirulina* for Enhanced Folate Production

For dry weight analysis, folate-producing bacteria and *Spirulina* were co-cultured to their respective growth phases. Daily, from day 1 to day 12, 1.5 ml samples were centrifuged to separate biomass from the medium. The supernatant was discarded, and the pellet washed to remove media residues. The dry weight of this cleaned pellet was measured on a balance to assess biomass productivity and growth performance, providing essential data for calculating specific growth rates and enhancing folate production.[16]

Harmonious Biosynthesis: Exploring Folate Quantification and Symbiotic Insights inMicroalgal-Bacterial Cultures

Folate levels in microalgal-bacterial cultures were determined by spectrophotometric analysis,[17] This entailed extracting folate from microalgal biomass via the phosphate buffer method and measuring absorbance at 282nm with a C-7200S double UV-visible spectrophotometer. A standard folate curve aided in precise measurement, with absorbance readings compared to the curve for calculating folate concentration. The symbiotic interaction between algae and bacteria in vitamin B_{12} acquisition, which is relevant to folate generation, was investigated.[18] This study investigated the process of folate synthesis in a microalgal-bacterial culture, with the goal of increasing folate production through symbiosis.

Statistical analysis

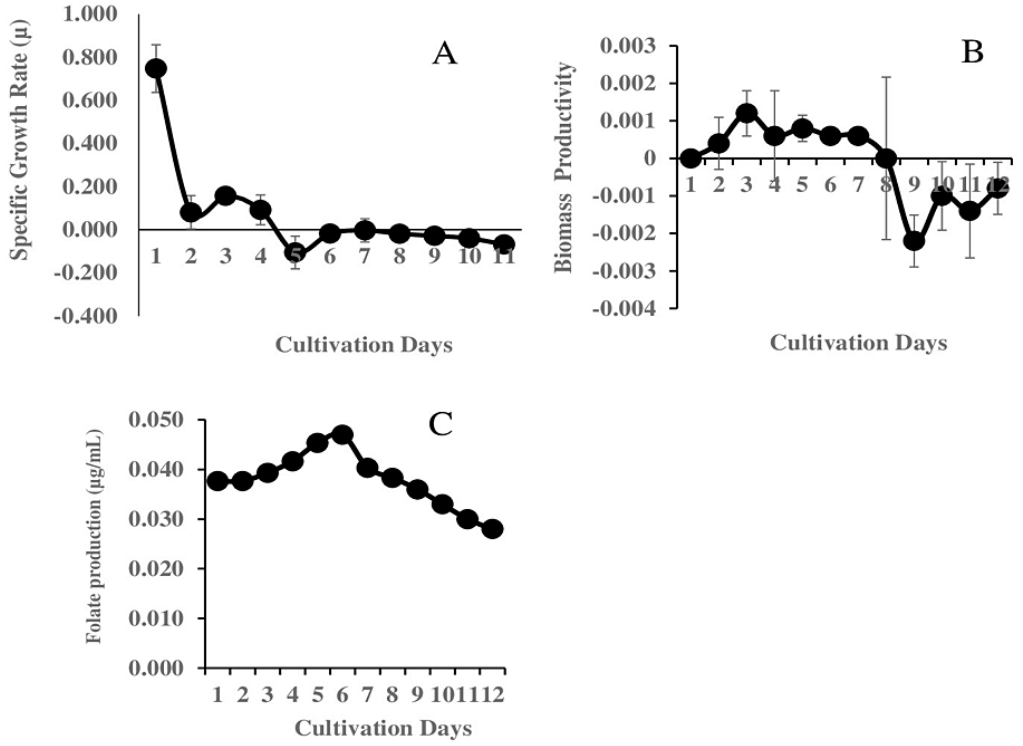

Fig.1. Dynamics for *Bacillus substilis*-1

A. Specific Growth Rate of *Bacillus substilis*-1. B. Biomass Produced. C. Folate Produced

The current data were presented in the form of average and SD. To know the difference between the groups the t tests, tests were applied by using Statistics version 9. The P values were considered significantly const.

Results

Table.1. Average Growth Dynamics and Folate Production in different phases of *Bacillus subtilis*-1

Phases	Treatment (Days)	Specific Growth Rate (μ)	Biomass Productivity	Folate production (μg/mL)
Lag	1	0.000±0.000	0.000±0.000	0.000±0.000
Log	2-5	0.269±0.321	0.001±0.000	0.043±0.003
Stationary	6-11	-0.036±0.036	-0.001±0.000	0.034±0.005
Decline	12	-0.069±0.000	-0.001±0.000	0.026±0.000

Table.2. Average Growth Dynamics and Folate Production in different phases of *Bacillus subtilis*-2

Phases	Treatment (Days)	Specific Growth Rate (μ)	Biomass Productivity	Folate production (μg/mL)
Lag	1	0.000±0.000	0.000±0.000	0.000±0.000
Log	2-3	0.446±0.399	0.001±0.000	0.041±0.002
Stationary	4-9	0.010±0.067	-0.001±0.002	0.041±0.005
Decline	10-12	-0.072±0.029	-0.002±0.002	0.034±0.001

Synergistic Growth and Folate Production of *Bacillus subtilis*-1

During the log phase (Days 2-5), *Bacillus subtilis*-1 exhibited a specific growth rate of 0.269±0.321 μ, biomass productivity of 0.001±0.000, and folate production of 0.043±0.003 μg/mL, suggesting active growth and metabolite synthesis. In the stationary phase (Days 6-11), a decline in specific growth rate to -0.036±0.036 μ was observed alongside decreased biomass productivity (-0.001±0.000) and slightly reduced folate production (0.034±0.005 μg/mL).Table 1, indicating a shift towards metabolic equilibrium. Fig. 1.

Unveiling Growth Dynamics and Folate Production of Bacillus Substilis-2

For *Bacillus Subtilis*-2, the organism showed robust growth in the log phase (Days 2-3) with a specific growth rate of 0.446±0.399 μ, minimal biomass productivity (0.001±0.000), and initiating folate production at 0.041±0.002 μg/mL. Entering the stationary phase (Days 4-9), growth significantly slowed (0.010±0.067 μ), and biomass productivity slightly declined (-0.001±0.002), yet folate production remained stable at 0.041±0.005 μg/mL. The decline phase (Days 10-12) witnessed a decrease in growth rate (-0.072±0.029 μ) Table 2, further reduced biomass productivity (-0.002±0.002), and a slight reduction in folate production to 0.034±0.001 μg/ml Fig.2.

For *Spirulina*, during the lag phase (Days 1-4), a specific growth rate of 0.212±0.182 μ was observed alongside biomass productivity of 0.041±0.028 and negligible folate production (0.000±0.000 μg/mL). In the subsequent log phase (Days 5-7), growth continued albeit at a slower rate, with a specific growth rate of 0.126±0.051 μ, biomass productivity of 0.026±0.009, and a minimal increase in folate production (0.001±0.001 μg/mL). During the stationary phase (Days 6-11), growth further slowed (0.045±0.045 μ), but biomass productivity increased slightly to 0.036±0.008, and folate production rose to 0.002±0.000 μg/mL. Finally, in the decline phase (Day 12), growth ceased (0.000±0.035 μ) Table 3, biomass productivity remained relatively constant (0.032±0.000), and folate production reverted to negligible levels (0.000±0.000 μg/mL) Fig. 3.

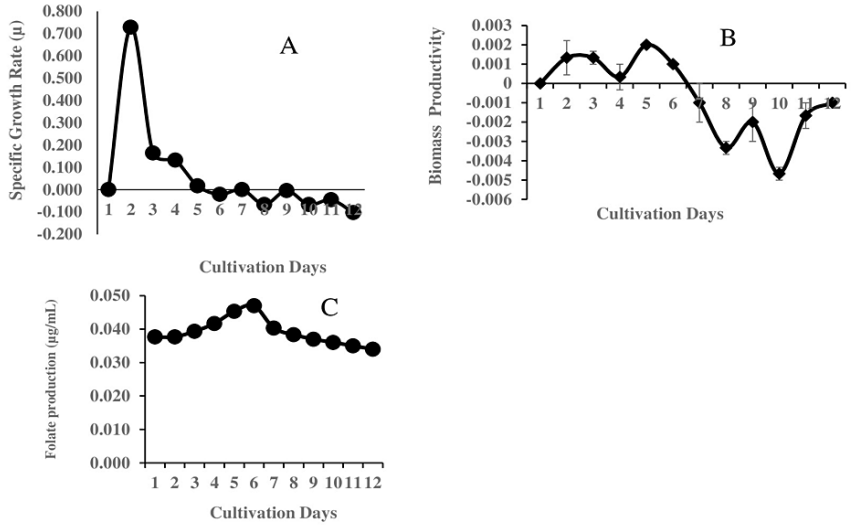

Fig.2. Dynamics for *Bacillus substilis*-2

A. Specific Growth Rate of *Bacillus substilis*-1. B. Biomass Produced. C. Folate Produced

Table.3. Average Growth Dynamics and Folate Production in different phases of *Spirulina*

Phases	Treatment (Days)	Specific Growth Rate (μ)	Biomass Productivity	Folate production (μg/mL)
Lag	1-4	0.212±0.182	0.041±0.028	0.000±0.000
Log	5-7	0.126±0.051	0.026±0.009	0.001±0.001
Stationary	6-11	0.045±0.045	0.036±0.008	0.002±0.000
Decline	12	0.000±0.035	0.032±0.000	0.000±0.000

Unveiling Synergistic Interplay between *Spirulina* and *Bacillus subtilis*-1 for Enhanced Folate Production

In co-culture, *Spirulina* and Bacillus Substilis-1 showed no growth or biomass productivity during the lag phase (Day 1), but an unusual initial folate production of 0.468±0.000 μg/mL was observed. During the log phase (Days 2-7), they exhibited a specific growth rate of 0.077±0.076 μ, minimal biomass productivity (0.001±0.002), and an increased folate production of 0.519±0.082 μg/mL. However, in the stationary phase (Days 8-11), a decrease in specific growth rate to -0.080±0.024 μ was noted alongside a slight decline in biomass productivity (-0.001±0.001) and a marginal reduction in folate production to 0.502±0.033 μg/mL Table 4. The decline phase (Day 12) saw all parameters reverting to zero, indicating cessation of growth and metabolite production Fig. 4

Unveiling the Synergistic Dynamics of *Spirulina* and Bacillus substilis-2 for Folate Enhancement

The co-culture of *Spirulina* with *Bacillus subtilis*-2 showed no initial growth or biomass productivity but had an early folate production of 0.468±0.000 μg/mL during the lag phase. The log phase witnessed an increase in specific growth rate to 0.188±0.147 μ and slight biomass productivity (0.001±0.002), with folate production rising to 0.519±0.074 μg/mL. In the stationary phase, a decline in specific growth rate (-0.029±0.043 μ) and a decrease in biomass productivity (-0.001±0.001) were observed, yet folate production remained relatively stable at 0.502±0.033 μg/mL Table 5. By the decline phase, all measured activities ceased, marking the end of the co-culture's growth cycle Fig. 5.

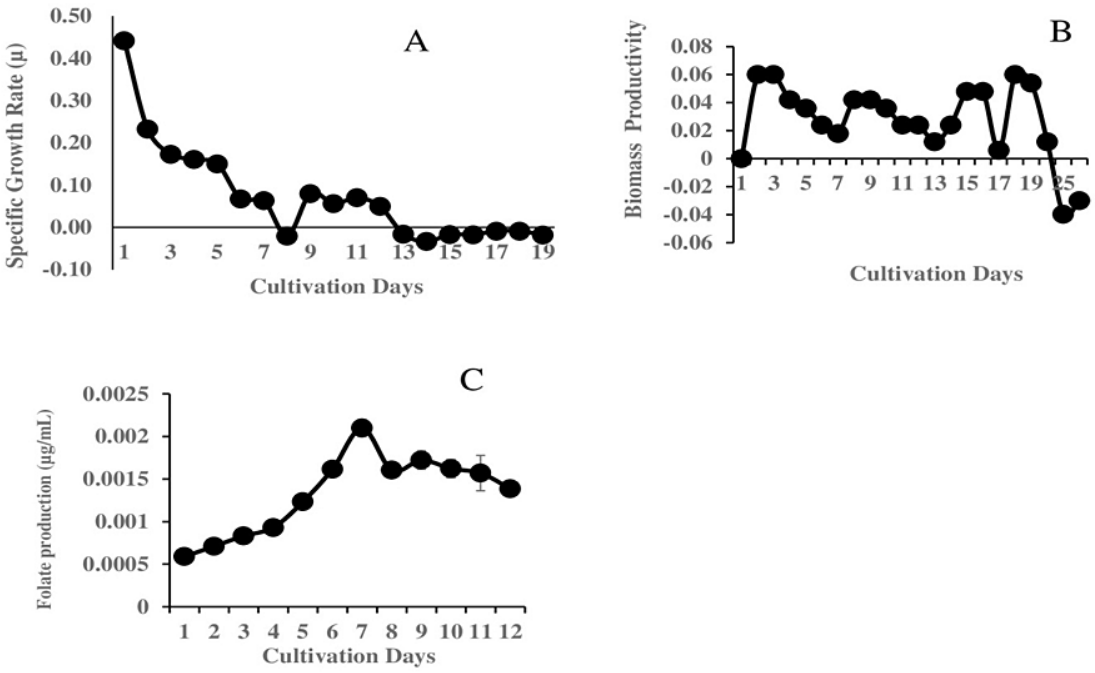

Fig.3. Dynamics for *Spirulina*

A. Specific Growth Rate of *Spirulina*. B. Biomass Produced. C. Folate Produced

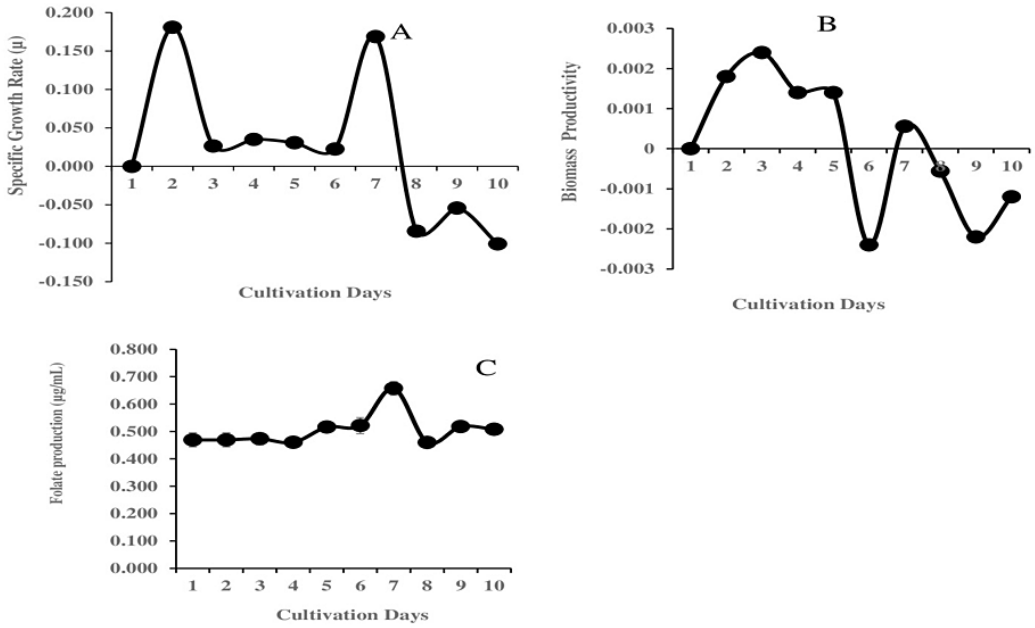

Fig.4.Synergistic Dynamics for Spirulina co-cultured with Bacillus substilis-1

A. Specific Growth Rate of Spirulina co-cultured with Bacillus substilis-1. B. Biomass Produced. C. Folate Produced

Table.4. Average Growth Dynamics and Folate Production in different phases of *Spirulina* co-cultured with *Bacillus substilis*-1

Phases	Treatment (Days)	Specific Growth Rate (μ)	Biomass Productivity	Folate production (μg/mL)
Lag	1	0.000±0.000	0.000±0.000	0.468±0.000
Log	2-7	0.077±0.076	0.001±0.002	0.519±0.082
Stationary	8-11	-0.080±0.024	-0.001±0.001	0.502±0.033
Decline	12	0.000±0.000	0.000±0.000	0.000±0.000

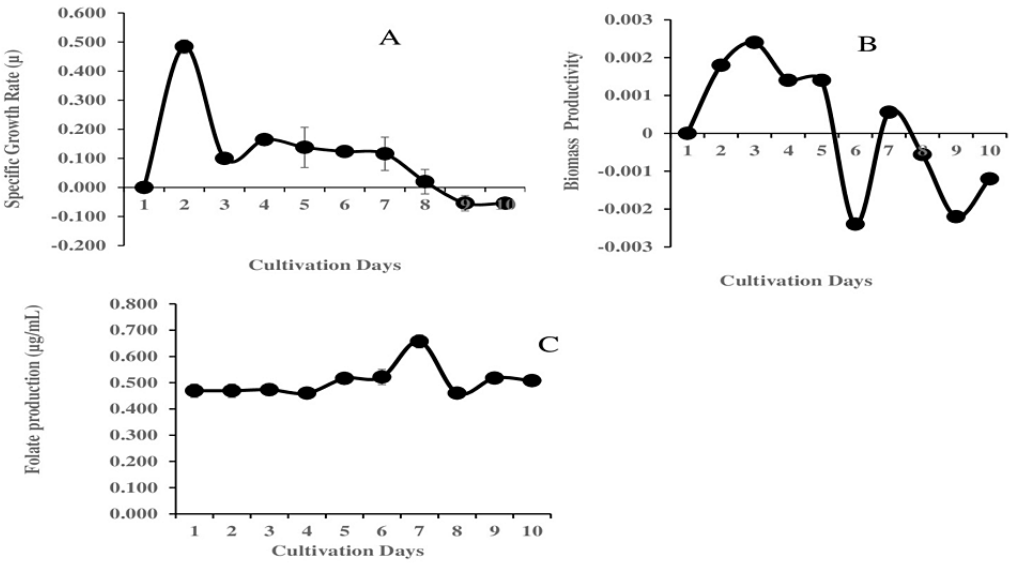

Fig.5.Synergistic Dynamics for Spirulina co-cultured with *Bacillus substilis*-2.
A. Specific Growth Rate of Spirulina co-cultured with *Bacillus substilis-2.* B. Biomass Produced. C.Folate Produced.

Table.5. Average Growth Dynamics and Folate Production in different phases of *Spirulina* co-cultured with *Bacillus substilis*-2

Phases	Treatment (Days)	Specific Growth Rate (μ)	Biomass Productivity	Folate production (μg/mL)
Lag	1	0.000±0.000	0.000±0.000	0.468±0.000
Log	2-7	0.188±0.147	0.001±0.002	0.519±0.074
Stationary	8-11	-0.029±0.043	-0.001±0.001	0.502±0.033
Decline	12	0.000±0.000	0.000±0.000	0.000±0.000

Discussion

The results presented in this study reveal significant insights into the growth dynamics and folate production of *Bacillus subtilis*-1, Bacillus Substilis-2, and *Spirulina*. The distinct growth phases observed in each microorganism played crucial roles in shaping their folate synthesis capabilities. Understanding these growth dynamics and their impact on folate production is essential for harnessing their potential in various applications. The exponential phase

observed in *Bacillus subtilis*1 showcased rapid growth and heightened folate synthesis. This phase is characterized by robust metabolic activities and efficient resource utilization, contributing to increased biomass and folate production. Similar findings have been reported in other folate-producing bacteria, emphasizing the significance of the exponential phase in enhancing folate synthesis.[19]

During the stationary phase of *Bacillus subtilis*-1, a balance between growth and sustained folate production was observed. This phase demonstrated stable growth conditions and a continued rise in folic acid production, indicating a consistent folate synthesis under favorable growth conditions. Other studies on Bacillus species have reported similar findings, suggesting that stationary phase cultures maintain folate production rates despites lower growth.[18, 20]

The decline phase in *Bacillus subtilis*-1 revealed the importance of optimal conditions for preserving folate synthesis. As the culture faced unfavorable conditions and nutrient limitation, both specific growth rate and folate production declined significantly. This reduction in metabolic activities and folate synthesis during the decline phase is consistent with observations in another Bacillus cultures.[21]

Moving on to Bacillus Substilis-2, the exponential phase exhibited a surge in folate synthesis despite a decline in growth rate. This phenomenon suggests a beneficial interplay between Bacillus Substilis-2 and the folate biosynthetic pathways, leading to enhanced folate production. The simultaneous increase in folic acid production and stability in biomass during this phase imply efficient nutrient utilization and metabolic activities.[22]

In the stationary phase of Bacillus Substilis-2, a balance between growth and folate production was maintained. The specific growth rate slightly decreased, but folate production remained relatively stable. This phase represents a period of adaptation and stabilization in the co-culture, allowing for the optimization of resource utilization and maintaining a balance between growth and folate.[23]

The decline phase in Bacillus Substilis-2 demonstrated reduced growth, metabolic activities, and folate production. The decline in specific growth rate and folate production during this phase can be attributed to various factors, such as nutrient depletion, altered microenvironment, or potential competition within the co-culture.[24]

Spirulina exhibited unique growth dynamics during the experiment. The exponential phase of *Spirulina* is characterized by rapid growth and increased metabolic activity, which plays a significant role in enhancing folate synthesis.[25] During the stationary phase of *Spirulina*, a balance between growth and folate production was observed. The specific growth rate decreased slightly, but the biomass remained constant, indicating a dynamic equilibrium between cell division and cell death. The steady rise in folate production during this phase suggests sustained folate synthesis under stable growth conditions.[26]

The co-cultivation of *Spirulina* with B. subtilis-1 and B. subtilis-2 revealed intriguing synergistic effects on folate production. Despite minimal growth and biomass productivity during the lag phase, an early and substantial increase in folate production was observed in both co-cultures. This suggests a potential cross-feeding or cooperative interaction between *Spirulina* and the Bacillus strains, enhancing folate biosynthesis. Furthermore, the fluctuations in specific growth rates and biomass productivity throughout the co-culture phases indicate dynamic metabolic interactions between the two organisms. Overall, the findings of this study shed light on the growth dynamics and folate production of the studied microorganisms, providing valuable insights into their potential applications in enhancing folate synthesis. Further research is warranted to explore the underlying mechanisms of the observed synergistic interactions and optimize the conditions for maximizing folate production in various industries.

Conclusion

Our research on *Spirulina* and folic acid-producing bacteria demonstrated synergistic interactions that increased folate synthesis across different growth stages. Co-culturing with *Bacillus subtilis*-1 and *Bacillus subtilis*-2, in particular, optimized growth and metabolic activities in the exponential phase while preserving cooperative contacts in the stationary phase for balanced symbiosis. The decline

phase showed growth constraints, emphasizing the significance of understanding these dynamics for better folate synthesis. This study highlights the potential of microbial synergy for sustainable nutrient production, laying the framework for future co-culturing techniques.

Recommendation

The outcomes of this study call for further investigation and application of co-culturing systems to maximize folate production. As we face global concerns like as population growth and rising demand for key nutrients, using microorganisms' collaborative capacity appears to be a possible road forward. The dance of growth and folate generation observed in *Spirulina* and its co-cultures demonstrates the beauty of microbial symbiosis. Moving forward, this understanding has the potential to inspire novel ideas and sustainable behaviors. Understanding and utilizing the synergistic interactions amongst microbes has the potential to revolutionize folate synthesis, contributing to a more nourished and thriving planet. The fascinating narrative of *Spirulina* and its microbial allies acts as a guiding ideal, stimulating study and collaboration for a healthier and more sustainable future.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

All the data obtained through experimentation are presented in this paper and further supplementary data could be retrieved from the corresponding author upon reasonable request.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

- **Maha Rehman:** Conceptualization, Methodology, Analysis, Writing
- **Rehan Naeem:** Conceptualization, Methodology, Review & Editing
- **Sunanda Biswas:** Visualization, Writing – Review & Editing
- **Maheen Sohail:** Methodology, Analysis, Data Collection
- **Sadeeq Khan:** Writing – Review & Editing
- **Muhammad Arif:** Methodology, Analysis, Data Collection
- **Nosheen Amjad:** Writing – Review & Editing
- **Saleha Tahir:** Writing – Review & Editing
- **Amina Mehmood:** Writing – Review & Editing

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