IN-SITU AND IN REAL TIME OBSERVATION OF PARTICULATE PROCESSES IN LACTIC FERMENTATION

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ARTICLE INFO

Received: 02/4/2024
Revised: 31/5/2024
Published: 31/5/2024

KEYWORDS

Particulate system
Lactic fermentation
Turbidity
Curcumin
Turmeric

ABSTRACT

Controlling bioprocesses requires understanding the behavior of bacterial populations, which necessitates real-time and in situ appropriate process monitoring techniques. Current market-available methods require various intermediate steps such as sampling, dilution, and measurement, which pose potential risks of contamination, in particular important for fermentation processes. To overcome these disadvantages, in this study, we develop a novel laser-based measurement that enables the continuously collection of bacterial population states every second under original conditions, without additional preparation steps. This innovative method allows collecting up to 25,000 to 30,000 measurement points, effectively capturing the growth, stationary, and decline phases of lactic bacteria as a case study. The robustness of the technique is evidenced by the excellent repeatability of duplicated experiments carried out under the same conditions. Additionally, via this novel method, the lactic fermentation process is observed that being significantly enhanced in the presence of turmeric and curcumin. In fact, these compounds reduce the dead rate of lactic bacteria, especially in the case of curcumin. Particularly, curcumin accelerates the growth and reproduction of *L. Bulgaricus*, which is in good agreement with results obtained from our developed equipment. Adding 2% (w/w) curcumin leads to an approximate 21.4% increase in the proliferation of the bacterial population. In short, this technique is highly recommended for monitoring particulate processes in biotech.

QUAN SÁT TRONG ĐIỀU KIỆN NGUYÊN BẢN VÀ THEO THỜI GIAN THỰC CÁC QUÁ TRÌNH TRONG LÊN MEN LACTIC

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THÔNG TIN BÀI BÁO

Ngày nhận bài: 02/4/2024
Ngày hoàn thiện: 31/5/2024
Ngày đăng: 31/5/2024

TỪ KHÓA

Quá trình hạt
Lên men lactic
Độ đục
Curcumin
Nghệ

TÓM TẮT

Điều khiển quá trình sinh học cần hiểu rõ hành vi của vi khuẩn, điều này yêu cầu cần áp dụng các kỹ thuật đo trong thời gian thực và điều kiện nguyên bản. Các phương pháp hiện có trên thị trường cần trải qua các bước trung gian như lấy mẫu, pha loãng, v.v. gây ra nguy cơ nhiễm khuẩn tiềm ẩn, đặc biệt đối với quá trình lên men. Để vượt qua các nhược điểm trên, trong nghiên cứu này, chúng tôi phát triển một phương pháp đo mới sử dụng laser nhằm thu thập thông tin liên tục về trạng thái quần thể vi khuẩn sau mỗi giây dưới điều kiện gốc mà không cần các bước trung gian. Phương pháp này cho phép thu thập từ 25.000 đến 30.000 điểm đo, cho thấy hiệu quả trong việc theo dõi các giai đoạn tăng trưởng, ổn định và suy giảm của vi khuẩn lactic. Độ ổn định của phương pháp thể hiện thông qua tính lặp lại của các thí nghiệm được thực hiện dưới cùng điều kiện. Ngoài ra, thông qua cách tiếp cận này, quá trình lên men lactic được chứng minh rằng có sự tăng cường khi có sự hiện diện của nghệ hoặc curcumin. Trong thực tế, các hợp chất này giảm tỉ lệ chết của vi khuẩn lactic, đặc biệt là curcumin. Curcumin giúp tăng cường quá trình sinh trưởng và phát triển của *L. Bulgaricus*, điều này phù hợp với kết quả đo được từ thiết bị được phát triển của chúng tôi. Cụ thể, việc sử dụng 2% curcumin có thể dẫn đến sự gia tăng của quần thể vi sinh vật lên 21,4%. Như vậy, phương pháp này rất phù hợp để nghiên cứu các quá trình hạt trong công nghệ sinh học.

DOI: https://doi.org/10.34238/tnu-jst.10020

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1. Introduction

Biotechnology plays a crucial role in the production of numerous high-value compounds such as essential acids, proteins, and vaccines, which can be efficiently obtained through fermentation processes [1] – [3]. Normally, the effective control of such intricate particulate processes is widely recognized as crucial in ensuring product quality. Therefore, the development of powerful monitoring techniques is considered a key task, which can provide real-time insights into the bio- and chemical reactions. This, in turn, facilitates immediate and precise process control. Unfortunately, conventional methods as described in the literature are still predominantly employed many offline techniques which involve intermediate steps such as sampling, dilution, preparation, and subsequent measurement [4], [5]. In these conventional procedures, crucial information may deviate significantly from the original state, posing challenges in maintaining process integrity. To diminish the risk of contamination and preserve the integrity of the process, there arises development of PAT (Process Analytical Technology) such as Focused Beam Reflectance Measurement (FBRM), 3D-ORM, etc. which needs for in-situ, real-time measurements applicable for monitoring particulate processes [6], [7]. Another challenge in bioprocesses involves the data acquisition that must meet statistical requirements. Extracting meaningful information from bioprocesses is inherently complex due to the presence of various types of noise. Therefore, relying solely on a few repeated measurements for each single point is often insufficient in many cases. Furthermore, the time intervals between data points pose another challenge, particularly when fast kinetic phenomena such as bio cell division is involved. For these reasons, in-situ and real-time techniques are again highly recommended. Each measurement must be captured within a very short interval without interfering the system.

The suggestion is gathering approximately few tens of thousand measurements per batch, to be able to statistically assess fermentation processes, applied for lactic as a case studied. In general, the life cycle of bacteria typically involves five stages including activation, growth, stationary, decline, and survival phases. In activation phase, bacteria enter a state of activity when conditions become favorable for growth. This can involve exposure to nutrients, appropriate temperature, and other environmental factors. Then in growth (logarithmic or exponential phase), bacteria multiply rapidly. The population size increases exponentially as they consume nutrients and divide at a high rate. This phase continues until nutrients become deplete or waste products accumulate, leading to a plateau in growth. Subsequently, in stationary phase, the growth rate of bacteria slows down and reaches equilibrium. The number of new cells produced equals the number of cells dying. Conditions such as limited nutrients or accumulation of waste products contribute to this phase. After that, in decline (death) phase, bacteria begin to die at a faster rate than they reproduce. This can occur due to nutrient depletion, accumulation of toxic by-products, or other unfavorable environmental conditions. The population size decreases rapidly during this phase. Finally, they reach to survival or dormant phase in which some bacteria have the ability to enter an inactive state to survive harsh conditions. They may form spores or cysts, which are highly resistant structures capable of withstanding extreme temperatures, desiccation, and exposure to chemicals or radiation. In this phase, metabolic activities are minimal, allowing the bacteria to persist until conditions become favorable for growth again. These stages of the bacterial life cycle can vary depending on the species of bacteria and the environmental conditions they encounter. Additionally, some bacteria may exhibit variations in their life cycle, such as the formation of biofilms or the ability to adapt to specific positions within their environment [8], [9].

Due to the fast kinetics of lactic bacteria, under optimized conditions, the growth in fermentation process could span about less than 1 day [10], [11]. In this period, lactic bacteria utilize substrates for growth, replication, and developing population size. When the available nutrients decrease, there comes a point where the death rate surpasses the birth rate, leading to a

decline in the population. The fluctuations in the population correspond to the change in turbidity of the system, which can be observed through a reduction in laser intensity as it continuously irradiates the reactor. Thus, relying on obtained laser signals, states of a fermentation process can be quickly captured. Thereby, effects of additives such as turmeric and curcumin could be assessed that provides different strategies for fermentation process control.

In this study, we constructed a specialized unit designed for in-situ ad in real time fermentation monitoring, which shares similarities to the unit employed for determining Solid-Liquid Equilibrium (SLE) and crystallization [12] – [14]. Our current researches have shown the effectiveness of the developed units in analyzing particle systems such as suspension and emulsion with extremely high concentrations. The current market technology like PVM (Particle Vision Measurement) or FBRM have limitation relating to defragmentation issues at elevated particle densities [15], [16]. In this work, we utilized a laser source emitting at 680 nm with a power of 5mW, which is suitable for detecting microorganisms, bacteria, and fungi. Especially, this method bases on a massive data acquisition related to more than 25,000 measurements per batch which allows monitoring processes in real time and in-situ conditions.

2. Experimental section

2.1. Materials

The bacterial strains used including *Lactobacillus bulgaricus LB-87 (L. bulgaricus)* and *Streptococcus thermophilus ST-21 (S. thermophilus)* in powdered form (10^9 CFU/g) provided by BioGreen Pharmaceutical and Biotechnology Joint Stock Company. KC-05 turmeric starch is a product researched and manufactured under the Research Project KC-05-07/06-10 of the Ministry of Science and Technology. The turmeric starch is extracted from the rhizomes of turmeric (*Curcuma longa* L.) originating from Hung Yen (Curcuminoid 4–6%, oil 1.5–2.5%, Ar-tumeron 1–1.5%, humidity 6–8%, total ash 5–6%.). Nano curcumin 20% from the Vietnam Academy of Science and Technology (mean particle size < 100 nm, curcumin 17.53%, demethoxycurcumin 2.8% and bisdemethoxycurcumin 0.97%).

2.2. Fermentation preparation

The study of curcumin or turmeric interaction was apllied simliar method to the literature [17]. First, prepare the MRS medium (Lactobacillus MRS Broth, Granulated, Himedia, India) according to the manufacturer's instructions: dissolve 55.15 grams in 1000 mL of distilled water, then sterilize at 121°C for 15 minutes. Subsequently, cool to 37°C using a water bath, inoculate lactic acid bacteria (bacterial concentration of 10^9 CFU/g, 3g/100ml) in the incubator 24h. Then, set the temperature to 37°C for fermentation and measurement of turbidity change in real-time using the turbidity measurement device. Record the turbidity measurements over time (collected per second) and plot the results on a graph. The morphology of bacteria were captured using SEM technique (Magnification of × 2,500 on TM4000plus, Hitachi, Japan). The nutrient in the media was frequently checked using Brix measurement [18]. Indeed, offline measurements were applied. 18 samples with the same composition were prepared in 6 seria. Parallel with the online sample, every 3 samples in the above 6 seria were objected to Brix measurement to evaluate the residual average nutrient after each of time interval.

In addition to the typical fermentation process involving equivalent mixture of *L. bulgaricus* and *S. thermophilus*, this study also examines the influence of curcumin and turmeric. There were supplements with 1% turmeric or 1% curcumin (and 2% curcumin) for investigation. Designing experiments were listed in Table 1 (all measurements were started after 10h of equilibrating with surroundings) with appropriate purposes including validation of repeatability, comparing effects of turmeric and curcumin on fermentation batches.

Table 1. *Experimental design plan*

Run	Conditions	Purpose
1st	Fermentation without additive	Repeatability validation
2nd	Fermentation without additive	Repeatability validation
3rd	Ferm. in presence of 1% turmeric	Study on turmeric effects
4th	Ferm. in presence of 1% curcumin	Study on curcumin effects
5th	Ferm. in presence of 2% curcumin	Study on turmeric dosage effects

2.3. Functionality of the laser-based equipment

Figure 1 graphically presents the basic construction and functionality of the turbidity measurement equipment. Briefly, the system (5) is placed in the sample holder (1), vial (6) is filled by fermentation media and positioned inside the thermal chamber (2). The stirring motor (7) is set at an appropriate speed to achieve homogeneous system and prevent gas bubbles. The temperature is gradually justified through the temperature control system and tungsten resistor (3). A laser beam cluster (4) with a wavelength of 680 nm and a power of 5 mW passes through the glass vial containing the fermentation media under investigation, with the focused beam directed onto the BH1750 sensor (10). The intensity of the laser beam received by the sensor module is transmitted to the micro processor and collected by the computer (9) via LabView. The internal temperature of the system is monitored using the Omron temperature sensor (8), which is also connected to the computer.

According to life cycle of bacteria, recorded laser will reflect number of bacteria variation. In this work, we measured turbidity of the fermenter which is a close function of number of cells.

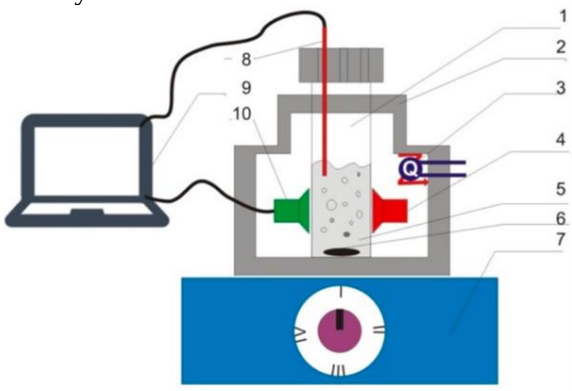

Figure 1. *In real time and in-situ turbidity measurement system* [12]

3. Results and discussion

3.1. Repeatability validation

Initially, two first experiments were conducted under identical conditions to verify the consistency of the measurement method. Generally, achieving repeatability in particulate processes (such as crystallization, emulsion formulation, fermentation, etc.) is extremely challenging. Numerous parameters influence variation even when processes are operated under the same conditions. An example can be introduced in the literature [19]. As another example, crystallization can be counted for complexity of a particulate process. According to Maggionia et al. [20], achieving consistent crystal size distributions of particle products from repeated crystallization driven by the same saturation degree is extremely difficult due to the stochastic nature of nucleation. Similarly, in fermentation processes, slight vibrations can cause variations in bacterial growth and reproduction, leading to divergent pathways in the overall process.

Figures 2a and 2b demonstrate the remarkable repeatability of the two validation batches. The observed trend indicates a continuous increase in population up to about 11,000 sec,

corresponding to the exponential growth phase of bacteria. Subsequently, the population decreases indicate the death phase which relates to nutrient depletion and an extremely acidic pH environment (pH = 3.4) due to bacteria activity. Thus, the developed measurement apparatus is robust to study lactic fermentation processes. Even though two batches presented in Figure 2 show very good agreement in their tendencies, discrepancy in these figure reveals the complexity of fermentation processes.

The same concept was applied for other particle processes such as SLE or crystallization which gained the same conclusions. The laser-based technology also works very well with materials such crystals of lactide, KDP, etc. [12] – [14]. Thus, the developed method is sustainable for variety of different particulate systems from bio cell to crystalline materials.

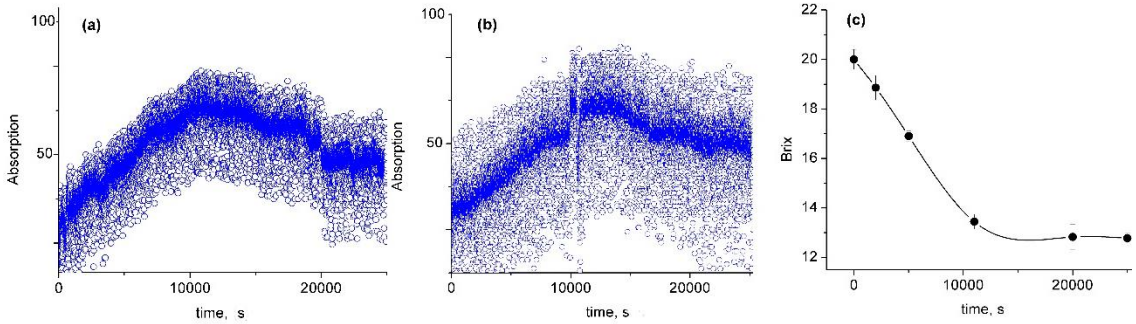

Figure 2. *(a) 1st, (b) 2nd fermentation processes with more than 25,000 points were collected per batch, (c) Residual nutrient evaluation via Brix measurement*

3.2. Investigation of lactic fermentation process

In general, lactic bacteria utilize nutrients for growth until a certain point in their life cycle, at which each bacterium divides into two daughter cells, resulting in exponential population growth. Towards the end of their life cycle, bacterial cell walls rupture to release internal contents, marking the onset of the death phase. Figure 3 predominants with the morphology of *S. thermophilus* bacteria, while *L. bulgaricus* seem to be lesser favor these conditions. In this depiction, the yellow marked – dashed line highlights *S. thermophilus* cell duplication, characterized by cell division into new cells, indicating growth following a power function as previously observed in Figures 2a and 2b. Additionally, the presence of dead cells with damaged walls and empty interiors, depicted in the red marked area, contributes negligibly to the interaction with incident laser rays. Consequently, the decrease in turbidity observed in Figure 2a and 2b corresponds to the death phase.

Figure 3. *Cell reproduction (yellow marked – dashed line) and cell death (red marked) morphology*

The subsequent experiment aimed to investigate the correlation between bacterial particles in the dispersion phase and the remaining nutrient in the mother liquid. As depicted in Figures 2a and 2b, the findings indicate a significant increase in cell numbers up to 11,000 sec, corresponding to the logarithmic phase wherein cells rapidly proliferate to establish an expanding population. The system reaches stationary phase between 11,000 and 12,500 sec. Subsequently, a decline in biomass occurred which indicates a death rate surpassing the birth rate. This phenomenon could be attributed to nutrient depletion and changes in environmental factors such as acidic pH and fermented by-product accumulate. At the same time, the residual quantity of nutrient was quantified, and its trend is illustrated in Figure 2c. The changes observed in both phases are in accordance with the principle of mass conservation. The nutrient in the liquid phase is utilized by bacteria and transformed into another form as bacterial population. Subsequent sections will investigate the variations in bacterial population under the presence of turmeric and curcumin.

3.3. Effects of turmeric on lactic bacteria activity

In the third run, the identical experimental conditions from the previous one were replicated but with the addition of 1% (w/w) turmeric powder, resulting in a distinct behavior of the system as depicted in Figure 4b. Notably, the growth phase appears to progress more rapidly compared to the previous case, indicating that the chemical compounds present in turmeric accelerate both in bacterial growth and reproduction. This observation aligns with similar conclusions reported in the literature [21]. Interestingly, in the presence of these compounds, the rate of cell death appears to be slower than in the previous run, as evidenced by comparing Figure 4a and 4b.

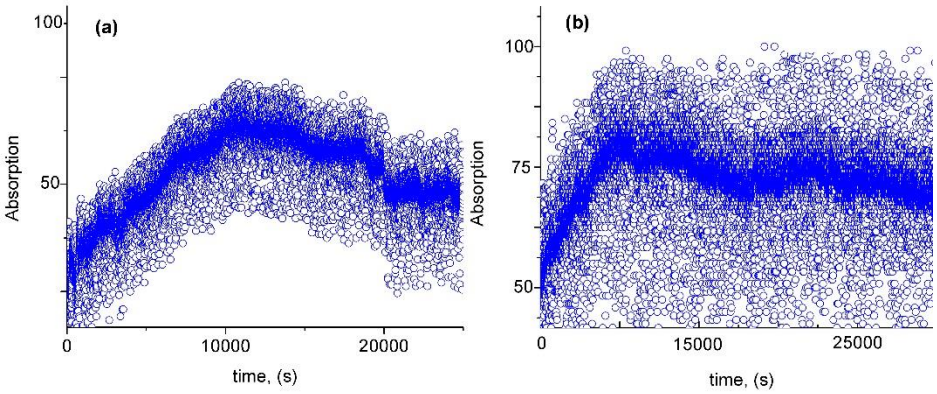

Figure 4. *Cell proliferation in fermentation processes (a) without additive and (b) using 1% turmeric*

In this particular instance, the data collected exhibited a notably higher level of scatter compared to the previous case. This discrepancy can be attributed to the presence of water-insoluble particles of turmeric suspending in the fluid. These particles occasionally interact with incident photon rays, resulting in scattered signals. This observation highlights a key advantage of the developed method, which relies on a large number of data points for statistical analysis, thus enabling robust assessments. Despite these sources of noise, the predominant trends during fermentation in the presence of turmeric remain distinctly apparent as seen in Figure 4b.

3.4. Effects of curcumin on lactic fermentation

Concerning the influence of curcumin, an absolute different situation was observed and presented in Figure 5. The first stage corresponding to growth phase is similar to the case of turmeric. The interesting point since 11,000 sec where the growth seems to be slow down but the dead phase affecting not significant anymore which results in a continuous growth even up to the end.

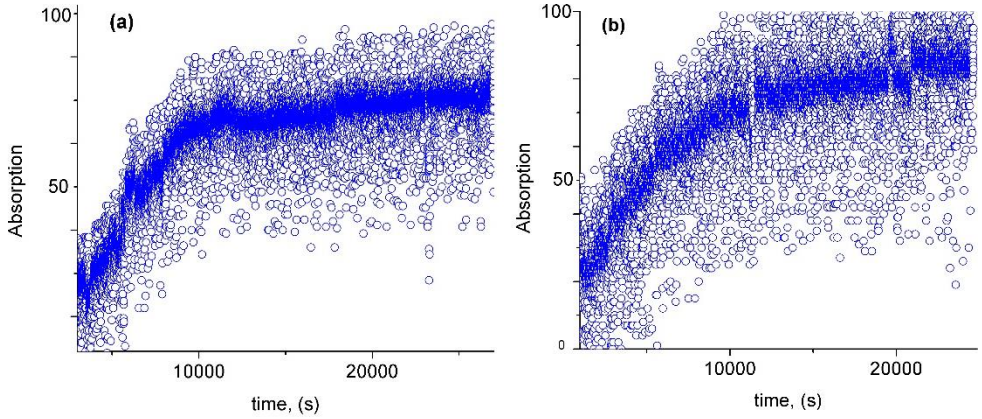

Figure 5. *Fermentation processes with the presence of (a) 1% curcumin, (b) 2% curcumin*

Curcumin, the active compound found in turmeric, has been proven to support the development of lactic bacteria. It could be resulted from several reasons. At first, curcumin exhibits antimicrobial activity against a wide range of bacteria, including harmful pathogens. By inhibiting the growth of competing bacteria, curcumin creates a favorable environment for the growth of lactic bacteria. Second, curcumin may act as a prebiotic, promoting the growth of beneficial bacteria. Prebiotics are non-digestible fibers that serve as food for probiotic bacteria, stimulating their growth and activity. Thus, curcumin provide a supportive environment for the proliferation of lactic bacteria.

Previously, there has been discussion on the supportive role of curcumin in the development of lactic bacteria populations. In this section, we conducted further investigation using a double quantity of curcumin and the result is illustrated in Figure 5b.

It is evident that birth and growth rates are influenced by the content of curcumin. An increase in curcumin to 2% results in approximately a 21.4% enhancement in the bacterial population proliferation comparing to normal fermentation without additives. Therefore, curcumin demonstrates its significance as a probiotic compound essential for lactic bacterial growth.

Notably, curcumin seems to promote the proliferation of *L. bulgaricus* bacteria over *S. thermophilus*. As demonstrated in Figure 3, *S. thermophilus* prevails in typical fermentation processes. However, the eventual population predominantly consists of bacteria displaying the characteristic morphology of *L.bulgaricus*, as depicted in Figure 6. This evidence emphasizes the selective impact of curcumin on different strains of lactic bacteria.

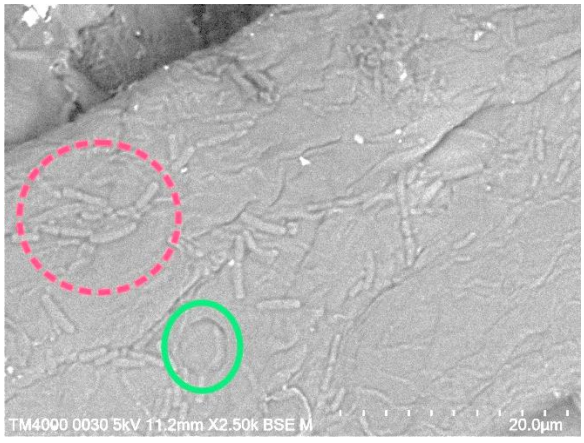

Figure 6. *L. bulgaricus (demonstrated in dashed-marked) is majority of the population after fermentation with presence of curcumin*

4. Conclusion

The complex phenomena of particle processes are interested, particularly in fermentation. Understanding the dynamics of bacterial behavior during different phases in their life cycle is crucial. These aspects have been the focus of numerous bioengineering studies over the years. In this paper, we present a novel self-engineering development leveraging laser technology, which enables the capture of significant processes in fermentation involving lactic bacteria. Our method demonstrates robustness, as evidenced by consistent results observed during repeated validation measurements. Moreover, it allows collecting data in real-time and in-situ conditions (1 sec time lapse) that is efficient for process control. Additionally, the abundance of collected data points facilitates the assessment of statistical phenomena associated with the studied bioprocesses. Through the application of this methodology, it was observed that curcumin facilitates the growth of lactic bacteria populations due to its antimicrobial, anti-inflammatory, prebiotic, and microbiota modulating attributes. These properties establish an environment conducive to the expansion and propagation of these advantageous bacteria. Indeed, inclusion of a small amount 2% (w/w) of curcumin could enhance 21.4% proliferation of lactic bacterial population. Similarly, turmeric exhibits comparable effects on lactic strains, however with less effectiveness than that of curcumin. For future work, the appropriate calibration curve will be constructed to convert population density into other quantified parameters such as CFU/g, etc. which definitely supports mathematical modeling efforts.

Acknowledgement

The authors would like to thank University of Technology and Education (Ho Chi Minh City, Vietnam) for their support to conduct this research. Besides, we also would like to thank Tu Trung Dan and Vo Thi Thuy Hang for their help in the experimental works.

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