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Effect of agitation rate for enhanced growth, metabolite production and kinetic modelling of *Bifidobacterium bifidum* fermentation

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ABSTRACT

Agitation rate is a critical parameter influencing microbial physiology, metabolite synthesis, and overall fermentation efficiency. This study investigated the effect of agitation rate on biomass growth, lactic acid production, and short-chain fatty acid (SCFAs) synthesis by *Bifidobacterium bifidum* TISTR 2129 in a stirred-tank bioreactor. Batch fermentations were conducted at agitation rates of 100, 300, and 500 rpm over 168 h using MRS broth under controlled microaerophilic conditions. The findings indicated that increased agitation significantly enhanced cell growth and lactic acid production. The maximum biomass concentration (5.8 g/L) and lactic acid yield (9.0 g/L) were achieved at 500 rpm, indicating enhanced substrate accessibility and activity by bacteria. SCFAs analysis indicated that the most varied and abundant metabolite profile was observed at 300 rpm, with lactic acid (2711.73 mg/L), acetic acid (618.77 mg/L), propionic acid (141.70 mg/L), and butyric acid (710.16 mg/L) identified. High agitation promoted fast biomass production, whereas moderate agitation (300 rpm) optimized lactic acid by ~52-fold compared to 100 rpm and by > 300-fold compared to 500 rpm. Based on the kinetic parameters presented for *B. bifidum* at different agitation rates (100, 300, and 500 rpm), the results of the fitted models (Monod, Logistic, and Gompertz) reveal several important insights into the microbial growth behaviour under varying oxygen transfer conditions. This study offers valuable information for the development of probiotic fermentation processes and facilitates the advancement of functional foods and pharmaceutical goods abundant in bioactive components.

Keywords: *Bifidobacterium bifidum* TISTR 2129, Agitation rate, Short-chain fatty acid, Monod model, Logistic model, Gompertz model, Bioreactor.

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1. INTRODUCTION

Probiotics, derived from the Greek meaning "for life," have studied for their beneficial roles in human health. While various definitions have been proposed, the Food and Agriculture Organization of the United Nations (FAO) formally defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health (Hill et al., 2014; McFarland, 2021), contributing to a wide range of health-promoting effects. Probiotics are predominantly associated with maintaining and enhancing gastrointestinal health. Consumption of probiotic-rich foods has shown to help prevent and mitigate disorders such as inflammatory bowel disease, diarrhea, and infections caused by *Helicobacter pylori,* a major contributor to gastric ulcers (Mestre et al., 2022; Vitetta et al., 2024). Beyond their role in gut health, probiotics strengthen immune responses and produce antimicrobial compounds that inhibit the proliferation of pathogenic microorganisms (Zhou et al., 2024). A diverse range of microorganisms function as probiotics, with acid-producing bacteria such as *Lactobacillus, Bifidobacterium*, *Streptococcus*, and *Lactococcus* being the most widely used in the food industry (Hossain et al., 2017). Non-acid-producing bacteria like *Propionibacterium* and *Acetobacter*, sporulating bacteria such as *Bacillus, Clostridium, Sporolactobacillus*, and *Brevibacillus*, and specific yeast strains including *Saccharomyces, Pichia, Issatchenkia*,

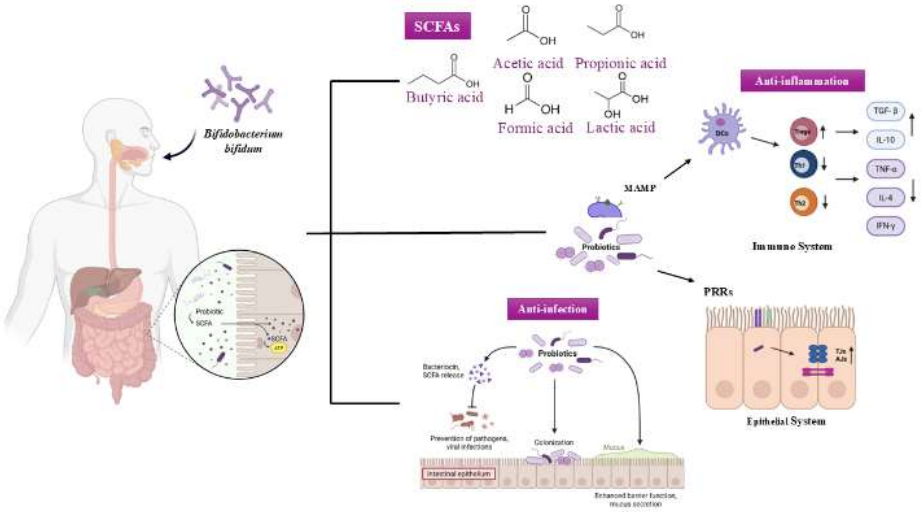


Fig. 1. Functions of *Bifidobacterium bifidum* in the intestine.

and *Kluyveromyces* have also demonstrated probiotic potential (Lee et al., 2024). Despite this diversity, acid-producing bacteria remains the dominant choice in probiotic food applications. With the increasing global emphasis on health, particularly following the COVID-19 pandemic, there has been a surge in consumer interest in functional foods, including probiotic-enriched products. The global market for probiotic foods is projected to reach approximately $94.5 billion by 2027, with an expected compound annual growth rate (CAGR) of around 7.90% between 2020 and 2027 (Fortune Business Insights, 2024).

Bifidobacterium bifidum, a crucial probiotic species, exhibits remarkable metabolic diversity and adaptability, making it a key player in gut microbiota balance. With an optimized genome for survival in the human digestive tract, *B. bifidum* relies on the bifid shunt pathway (Devika and Raman, 2019; Chen et al., 2021), efficiently metabolizing carbohydrates to produce beneficial short-chain fatty acids such as acetate and lactate. For *B. bifidum* can utilize glucose, fructose, sucrose and lactose but not ribose, arabinose, cellobiose, sorbitol, xylose and mannose (Xiao et al., 2024). These metabolites contribute to intestinal health by supporting microbiome stability and influencing host immune responses. Industrially, *B. bifidum* is widely utilized in probiotic formulations, functional foods, and nutraceuticals, owing to its role in maintaining gastrointestinal health and exhibiting anti-inflammatory properties (Sun et al., 2020; Latif et al., 2023). Its evolutionary position, shaped by niche-specific adaptations, highlights its genetic flexibility through an open pan-genome, enabling strain-specific functional specialization. The versatility of *B. bifidum* emphasizes its potential for advancing probiotic research and biotechnological applications.

Understanding the metabolic behavior and growth dynamics of *B. bifidum* is essential for optimizing its use in industrial processes, particularly in microbial fermentation. The study of microbial fermentation processes and metabolite production plays a pivotal role in biotechnological research, particularly in the development of organic acids, enzymes, and other valuable biomolecules, as shown in Fig. 1. Given the complexity of microbial growth, mathematical modelling has emerged as an indispensable tool for analysing experimental data, offering insights into cell growth, substrate consumption, and product formation.

The agitation rate plays a crucial role in probiotic activation. An appropriate agitation rate enhances nutrient distribution and mass transfer, thereby boosting viable cell concentration and overall productivity. However, excessively high agitation can exert shear stress on cells, potentially leading to cell damage or after metabolite production (Baral et al., 2021; Valle-Vargas et al., 2025). By employing mathematical models, researchers can significantly reduce the need for extensive laboratory experimentation under varied conditions, thus minimizing research time and costs. Among the various models applied in microbial growth analysis, the Monod Model serves as a foundational framework for describing microbial proliferation under substrate-limited conditions. This model quantifies microbial growth rates as a function of nutrient availability, offering valuable predictions for large-scale fermentation processes where substrate concentrations fluctuate. In addition, the Gompertz Model helps describe the asymmetric sigmoid growth patterns of microbial populations, making it particularly useful for predicting bacterial proliferation within resource-limited environments. Complementing these models, the Logistic Model provides a structured approach to evaluating microbial growth dynamics in constrained conditions, demonstrating how populations slow their expansion as they near environmental carrying capacities. This rising demand has fueled research focused on improving the efficiency of probiotic biomass production. Optimizing probiotic

biomass production requires careful control of key parameters, including the pH of the growth medium, fermentation temperature, and agitation rates within bioreactors. By refining these factors, researchers aim to maximize the yield of biomass production of probiotic *B. bifidum* using a stirred tank bioreactor with agitation rates of 100, 300 and 500 rpm. Kinetics of growth by monitoring the changes of biomass, glucose and lactic acid were evaluated and reported by using Monod, Logistic and Gompertz model. Overall, this study provides new insights into how agitation rate influences both growth kinetics and metabolite diversity of *B. bifidum*, establishing a framework for scalable probiotic fermentation in functional food and biopharmaceutical applications.

2. MATERIALS AND METHODS

2.1 Chemicals

3,5-Dinitrosalicylic acid was obtained from LOBA Chemie, Mumbai, India. Medias, De -man, rogosa and sharpe (MRS broth and agar), were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Nutrient broth and nutrient agar were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India). 2,2-Diphenyl 1-picrylhydrazyl was brought from Sigma-Aldrich, Missouri, USA.

2.2 Strain and Growth Medium

Freeze-dried *B.bifidum* TISTR 2129 were obtained from Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand. Bacteria strain was inoculated into MRS media and incubated at 37ºC under anaerobic condition, using a anaeroPack®-Anaero (Mitsubishi gas chemical Co., Inc, Japan) as oxygen-absorbing and carbon dioxide-generating agent for 48 h.

2.3 Inoculum Preparation

B.bifidum TISTR 2129 from stock cultures were transferred to sterile test tube containing 5 mL MRS media. The cultures were incubated at 37ºC without agitation for 72 h under anaerobic conditions. The cultures broth was transferred to an Erlenmeyer flask containing 50 mL of medium and a starter was incubated at 37ºC for 72 h under anaerobic conditions before further transfer to sterile MRS media for preparation 10% inoculum (500 mL culture media) as a seed for 5 L fermentation.

2.4 Effect of Agitation Rate on Biomass and Lactic Acid Production

To perform the scale-up of biomass production in a bioreactor, an inoculum size of 10 percent of cultures was inoculated into a flask culture with a 500 mL working volume, respectively. Batch cultivations in a stirred tank bioreactor were carried out using a 7.5-L laboratory-scale stirred tank bioreactor (Bioflo120, Eppendorf, Inc., Enfield, CT, USA) with a working volume of 5 L. The MRS broth was sterilized at 121°C for 15 min, cooled, and then inoculated with inoculum. 500 mL of inoculum of *B. bifidum* TISTR 2129 was inoculated in a bioreactor containing 5L MRS broth. The temperature was controlled at 37°C, and the agitation rate was maintained at 100, 300 and 500 rpm throughout the cultivation. The stirrer was equipped with two 6-bladed Rushton turbine impellers. Dissolved oxygen was maintained < 0.5 mg/L via headspace nitrogen flushing prior to inoculation and inclusion of reducing agents in MRS broth, with continuous monitoring using a polarographic dissolved oxygen probe (Ingold, Mettler-Toledo, Switzerland). The initial medium pH was adjusted to 6.0 and then maintained by cascading the pH controller with acid/base feeding peristaltic pumps connected with 3.0 M HCl and 3.0 M NaOH, respectively. Samples of fermentation were taken every 24 h for analysis.

2.5 Sampling and Analytical Analysis

5 mL samples of the fermentation medium were collected at predetermined intervals and centrifuged at 5,000 rpm for 10 min. The resulting precipitate was washed twice with 5 mL of distilled water, then gathered and dried at 90°C until a stable weight was achieved. The obtained supernatant was used to determine quantification of reducing sugar using Dinitrosalicylic acid method (Miller, 1959). Lactic acid concentration in fermentation broth was quantified by titration method. All measurements were done in triplicate. The key experimental rates, including biomass production rx, where (r_x = d[X]/dt) and (X) represents biomass concentration), lactic acid formation rp, where (r_p = d[P]/dt) and (P) is lactic acid concentration), and glucose consumption r_s, where (r_s = d[S]/dt) and (S) denotes glucose concentration) were calculated from the experimental data over 168 h fermentation.

2.6 Short Chain Fatty Acid Production

The evaluation of short-chain fatty acids (SCFAs) produced by *B. bifidum* TISTR 2129 was performed using culture supernatant obtained after 168 h of fermentation. The fermentation broth received centrifugation to separate the biomass, and the resulting supernatant was filtered using a 0.22 µm syringe filter (Merck Millipore, Germany). The filtered supernatant was combined with 99% methanol in a 1:5 (v/v) ratio, then exposed to centrifugation at 4,000 rpm for 10 min at 4°C to precipitate proteins. The emptied supernatant underwent an examination via high-performance liquid chromatography (HPLC) utilizing a Rezex ROA-Organic Acid H^+ column (300 × 7.8 mm; Phenomenex, USA) maintained at 40°C. The mobile phase comprised 0.005 M sulfuric acid at a flow rate of 0.6 mL/min. SCFAs were recognized and determined with the Shimadzu Nexera LC-40, Japan as acetic, lactic, butyric and propionic acids (Usta-Gorgun and Yilmaz-Ersan, 2020).

2.7 Mathematical Modelling

The concentration profiles of biomass, production of

aeration rate at 100, 300 and 500 rpm observed in batch experiments were modelled used for analysing biological behaviour. Three mathematical models, Monod model, Gompertz Model and Logistic Model were applied to fit the experimental data of the fermentation process. Kinetic parameters were determined through examination of experimental profiles and their corresponding production Modelling validity was confirmed using three independent sets of experimental data encompassing biomass production. Three model equations were defined in Equations (1–3).

The Monod Model is expressed by the following equation:

$$\mu = \mu_{max} \cdot \frac{S}{K_S + S} \quad (1)$$

Where: μ is the specific growth rate of the microorganism (h^{-1}). μ_{max} is the maximum specific growth rate, representing the highest growth rate when nutrients are unlimited (h^{-1}). S is the substrate concentration in the environment (g/L). K_s is the substrate saturation constant (g/L), indicating the substrate concentration at which the growth rate is half of the maximum growth rate.

The equation for the Gompertz Model is given by:

$$\ln\left(\frac{X}{X_0}\right) = a\ exp\ [-exp(b - ct)] \quad (2)$$

Where: X represents the cell concentration at time (g/L). X_0 is the initial initial cell concentration (g/L). a is a constant associated with the maximum growth rate (h^{-1}). b is a constant that defines the starting point of growth (h^{-1}). c is a constant related to the rate of growth slowdown (h^{-1}). t is the time (h).

The Logistic Model represents a symmetric sigmoid growth pattern, where the growth rate is dependent on the population size and becomes limited as the system approaches its maximum capacity. The growth rate decreases as the population approaches the carrying capacity of the environment. This model is commonly used in studies of population dynamics where the growth is constrained by resource limitations (Tsikliras and Froese, 2019).

The equation for the Logistic Model is as follows:

$$\ln(\frac{X}{X_0}) = \frac{a}{\mathbf{1-exp(b-ct)}} \quad (3)$$

where: X is the cell concentration at time (g/L). X_0 is the initial cell concentration (g/L). a is a constant that reflects the difference between the maximum population size and the initial population size. b is a constant that relates to the initial growth rate (h^{-1}). c is a constant that defines the rate of growth slowdown (h^{-1}). t is the time (h).

This model is particularly useful for describing growth where the population initially grows rapidly and then slows as it nears the environmental capacity, providing a clear picture of the dynamics of limited growth.

3. Results and Discussion

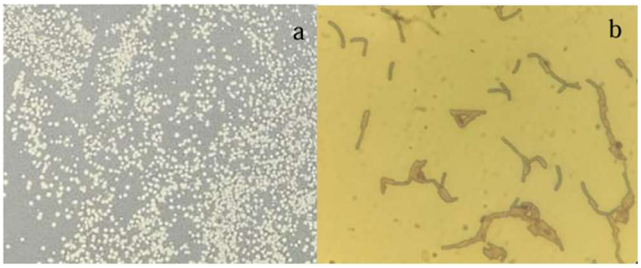
3.1 Morphological characteristics of *B.bifidum* TISTR 2129

The cultural and morphological characteristics of *B. bifidum* TISTR 2129 (Original no. DSM 20456, ATCC 29521 and JCM 1255) were observed on MRS agar using light microscopy. As shown in Fig. 2(a), colonies grown on MRS agar appeared as small, round, creamy-white, and convex with entire margins, which are typical characteristics of Bifidobacterium species. When examined using a 100x objective lens, the bacterial cells exhibited a rod shape with slightly curved and bifurcated sides as shown in Fig. 2(b). These morphologies are consistent with previously reported characteristics of *B.bifidum*, particularly strain ATCC 29521, which is known for its branched rod shape (Segui-Perez et al., 2025). This identification is significant, as *B. bifidum* ATCC 29521 has been shown to play a protective role in ulcerative colitis, especially in dextran sodium sulfate (DSS)-induced colitis models.

The strain's well-documented benefits such as immune modulation, reinforcement of the gut barrier, oxidative stress reduction, microbiome restoration, and microRNA interaction underscore the importance of its accurate morphological confirmation for future therapeutic applications. These morphological confirmations not only align with the established characteristics of *B. bifidum* ATCC 29521 but also support its potential as a functional probiotic strain with therapeutic relevance. Previous studies have demonstrated that this strain exerts protective effects against DSS-induced colitis through several synergistic mechanisms. It modulates the host immune response by enhancing anti-inflammatory cytokines such as IL-10, $PPAR_{\gamma}$, and IL-6, while downregulating pro-inflammatory mediators including TNF-α and IL-1β (Din et al., 2020; Xiao et al., 2024). Furthermore, this strain plays a crucial role in maintaining gut epithelial integrity by upregulating key tight junction proteins ZO-1, MUC-2, Claudin-3, and E-Cadherin-1which are vital for sustaining the mucosal barrier.

In addition to its immunomodulatory and barrier-enhancing functions, this probiotic bacterium contributes to oxidative stress mitigation by stimulating the activity of reactive oxygen species (ROS)-scavenging enzymes such as SOD1, SOD2, CAT, and GPX2. These antioxidant responses help attenuate inflammation-induced tissue damage. Notably, the strain also aids in the rebalancing of gut microbiota disrupted by DSS exposure, indicating its role in microbiome restoration (Din et al., 2020; Weng et al., 2021). Recent evidence further reveals that *B. bifidum* ATCC 29521 can modulate host microRNA expression-specifically miR-150, miR-155, and miR-223 which are involved in fine-tuning inflammatory pathways (Din et al., 2020). Collectively, these findings underscore the multifunctional nature of this strain and its potential utility

as a probiotic intervention in the management of ulcerative colitis.



(a) (b)

Fig. 2. Cultural characteristics of *Bifidobacterium bifidum* TISTR 2129 grown on (a) MRS agar and (left) (b) microscope with a 100x objective lens (right).

3.2 Effect of Agitation Rate on Biomass and Lactic Acid Production by *B.bifidum* TISTR 2129 in Bioreactor

The biomass dry weight increased when the agitation rate increased from 100 to 500 rpm was presented in Table 1. By enhancing the mass transfer characteristics of substrates and products, agitation rate may be advantageous to the development and performance of microorganism cells. Thus, agitation improves the mixing of fermentation broth, so assisting in the maintenance of a concentration gradient between the interior and outside of the cells. In addition, agitation rate helps maintain a sufficient supply of sugars and other nutrients to the cells which is essential for a high biomass concentration. In cultures agitated at 500 rpm, the maximum biomass and lactic acid concentration were shown after 168 h of fermentation (5.8 g/L and 9.0 g/L respectively). The biomass in cultures agitated at 100 and 300 rpm were 2.8 g/L and 4.8 g/L, respectively. The yields of cell mass ($Y_{x/s}$) were 0.21 g/g, 0.36 g/g and 0.33 g/g respectively, at agitation rates of 100, 300 and 500 rpm, this suggests that moderate agitation enhanced mass transfer possibly improving substrate and nutrient distribution without introducing lethal levels of dissolved oxygen. It is likely that the actual dissolved oxygen concentration remained within the microaerophilic range (e.g., $\leq 0.5\%$ O_2), particularly if reducing agents were included in the medium. However, at 500 rpm, although the $Y_{x/s}$ remained relatively high at 0.33 g/g, there was no significant gain in biomass compared to 300 rpm. This plateau or potential decline suggests that at higher agitation, the oxygen transfer rate may have approached or exceeded the oxygen tolerance threshold of *B. bifidum*, resulting in oxidative stress that limited further growth. The data imply that 300 rpm was likely the optimal agitation rate under these conditions, balancing sufficient mixing and substrate availability without inducing oxygen toxicity.

The rate of agitation improved the uptake of sugars. This was consistent with the increased biomass and lactic acid concentration when agitation speed increased. At reaching the maximal concentration of lactic acid in the broth for cultures agitated at 100, 300 and 500 rpm, the quantities of glucose utilized were 51.76, 63.78 and 79.33% respectively. The fact that both biomass and lactic acid continued to increase at 500 rpm suggests that oxidative stress had not yet reached inhibitory levels, or that the strain and medium conditions provided sufficient protection (e.g., reducing agents). Nonetheless, the diminishing returns in cell yield observed in the previous data suggest that further increases in agitation could eventually have a negative impact. Changes of dry cell weight, residue sugar concentration and lactic acid concentration at various agitation speeds are shown in Fig. 3–5. To analyse the kinetic characteristics of these processes, kinetic parameters including maximum specific growth rate specific sugar consumption rate and cell mass productivity were calculated based on the data of Table 1. The effect of agitation rate on the growth kinetics of *B. bifidum* ATCC 29521 was evaluated by monitoring the maximum specific growth rate at 100, 200, and 300 rpm. In Table 2 the change was shown the maximum specific growth rate, specific sugar consumption rate and cell mass productivity within the fermentation process. When increase of agitation speed resulted in a quick start of growth (shortened lag time) and increase of biomass formation. The highest dry cell weight (5.8 g/L) was achieved at 168 h at the agitation speed of 500 rpm, as shown in Fig. 5. Correspondingly, it was found that the higher the agitation speed, the higher the maximum specific growth rate, as shown in Table 2. A relatively high agitation speed was also favourable for sugar consumption. The specific sugar consumption rate shows a similar tendency to maximum specific growth rate, and a relatively high specific sugar consumption rate was achieved at high agitation speed.

As shown in Table 2 and Fig. 6, increasing the agitation rate from 100 to 300 rpm resulted in a substantial rise in the specific growth rate, from 0.23 h^{-1} at 100 rpm to 0.37 h^{-1} at 200 rpm, and reaching a peak of 1.21 h^{-1} at 300 rpm. This trend indicates that enhanced mixing at higher agitation rates significantly promoted bacterial growth. The observed increase in growth rate attributed to improved mass transfer of nutrients, as agitation enhances convective mixing in the medium. For anaerobic or microaerophilic bacteria like *B. bifidum*, a moderate increase in oxygen availability can support critical biosynthetic pathways and energy production, without reaching inhibitory levels. Additionally, higher agitation likely minimized local nutrient gradients, providing a more homogeneous environment for cell proliferation. However, it is essential to consider that excessive agitation beyond optimal levels can lead to shear stress, which may negatively impact the integrity of sensitive probiotic strains. In this study, 300 rpm appears to fall within the optimal agitation window for this bacterium without observable detrimental effects. The relationship between agitation rate and the doubling time of *B. bifidum* was investigated in a stirred tank bioreactor operated at 100, 200, and 300 rpm. As presented in Table 2, the doubling

Table 1. Effect of agitation rate on biomass and lactic acid production by *Bifidobacterium bifidum*

Fermentation time (h)	Agitation rate (rpm)	Absorbance 600 nm	C_x (g-cell/L)	C_s (g-glucose/L)	Lactic acid concentration (g/L)
0	100	0.226 ± 0.01	0.8	19.80 ± 0.33^{b}	1.80 ± 0.00^{a}
	300	0.166 ± 0.02	0.2	19.77 ± 1.48^{c}	2.40 ± 1.04^{b}
	500	0.165 ± 0.01	0.4	20.37 ± 1.37^{a}	2.40 ± 1.04^{b}
24	100	0.237 ± 0.01	1.0	16.41 ± 0.35^{b}	1.80 ± 0.00^{b}
	300	0.167 ± 0.02	0.4	19.09 ± 1.36^{a}	3.00 ± 1.04^{ab}
	500	0.555 ± 0.03	2.6	10.31 ± 0.38^{c}	4.20 ± 1.04^{a}
48	100	0.628 ± 0.00	1.6	15.22 ± 0.06^{b}	2.70 ± 0.09^{b}
	300	0.799 ± 0.02	1.0	17.49 ± 0.24^{a}	6.60 ± 1.04^{a}
	500	1.111 ± 0.00	3.0	4.59 ± 0.38^{c}	4.80 ± 2.08^{ab}
72	100	0.761 ± 0.00	2.2	14.67 ± 0.19^{a}	3.60 ± 0.00^{b}
	300	1.163 ± 0.01	2.4	10.31 ± 0.54^{b}	7.80 ± 1.04^{a}
	500	1.536 ± 0.03	3.2	4.45 ± 0.38^{c}	6.00 ± 0.00^{a}
96	100	0.768 ± 0.00	2.2	13.83 ± 0.07^{a}	3.60 ± 0.00^{b}
	300	1.292 ± 0.02	2.4	10.02 ± 0.20^{b}	7.80 ± 1.04^{a}
	500	1.677 ± 0.01	3.4	4.40 ± 0.88^{c}	7.20 ± 1.04^{a}
120	100	0.842 ± 0.00	2.2	13.34 ± 0.10^{a}	3.60 ± 1.04^{b}
	300	1.299 ± 0.02	3.6	7.53 ± 0.15^{b}	8.40 ± 1.04^{a}
	500	1.698 ± 0.00	4.6	4.36 ± 0.38^{c}	7.80 ± 1.04^{a}
144	100	0.846 ± 0.00	2.4	13.05 ± 0.02^{a}	4.20 ± 1.04^{b}
	300	1.311 ± 0.01	4.0	7.20 ± 0.02^{b}	8.40 ± 1.04^{a}
	500	1.732 ± 0.00	5.2	4.29 ± 0.04^{c}	7.80 ± 1.04^{a}
168	100	0.940 ± 0.00	2.8	9.55 ± 0.10^{a}	4.20 ± 1.04^{b}
	300	1.352 ± 0.01	4.8	7.16 ± 0.09^{b}	8.40 ± 1.04^{a}
	500	1.726 ± 0.00	5.8	4.21 ± 0.19^{c}	9.00 ± 0.00^{a}

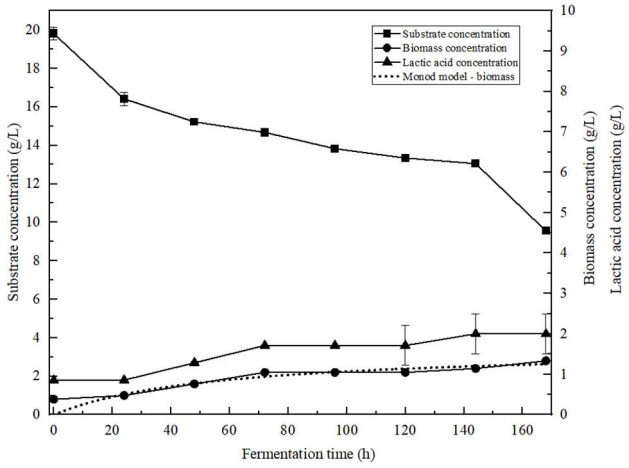
Note: mean ± SD, a-c means within each column indicate significant differences ($P < 0.05$) using ANOVA analysis.

Table 2. Summary of *Bifidobacterium bifidum* TISTR 2129 growth kinetics data for batch

Agitation rate (rpm)	μ_{max} (h^{-1})	T_D (h)	$Y_{x/s}$ (g-cell/g-glucose)	$Y_{p/s}$ (g-lactic acid/g-glucose)	Productivity (g/g-h)
100	0.23	70.51	0.21	0.23	0.01
300	0.37	44.31	0.36	0.33	0.02
500	1.21	13.71	0.33	0.40	0.03

time decreased significantly with increasing agitation, from 70.51 h at 100 rpm to 44.31 h at 200 rpm, and further to 13.71 h at 300 rpm. This inverse correlation indicates that higher agitation rates substantially enhance bacterial growth rates, consistent with the corresponding increase in specific growth rates (μmax = 0.23, 0.37, and 1.21 h^{-1}, respectively).

The reduction in doubling time with increasing agitation attributed to several factors. Primarily, enhanced mixing at higher speed improves the distribution of nutrients and the removal of metabolic byproducts, creating a more favourable environment for cell division. Research by De Bruyn et al. (2024) suggests that agitation affects nutrient distribution, which directly influences fermentation efficiency and metabolite yields. Furthermore, studies show that moderate agitation rates (such as 300 rpm) optimize nutrient availability, which promotes higher microbial yields and metabolite diversity (Lee et al., 2024; Xiao et al., 2024). Although *B. bifidum* is a microaerophilic organism, moderate increases in oxygen transfer rates due to agitation likely support its metabolic activity without causing oxidative stress. Moreover, improved hydrodynamic conditions may minimize concentration gradients, thereby preventing nutrient limitation zones in the culture medium. However, with respect to lactic acid production, the lactic acid concentration increased when the agitation speed was 500 rpm but decreased if the agitation rate was decreased. The visual representation of the fermentation broths (Fig. 6) corroborates these observations. At reduced agitation rates (100 rpm), the broth maintained a darker and more turbid appearance over the incubation time, indicating constrained microbial growth and metabolic activity. In contrast, broths cultured at 300 and 500 rpm exhibited a gradual darkening of colour, particularly after 72 h, indicating increased substrate use and biomass production.



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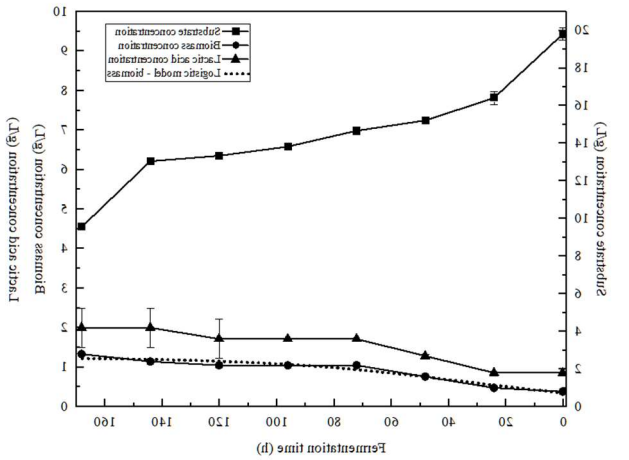

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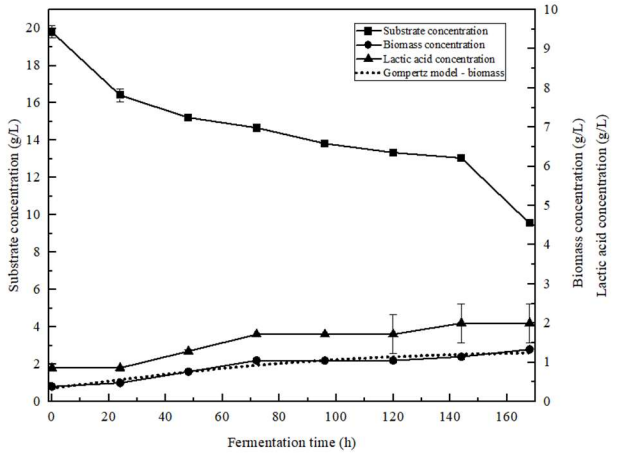
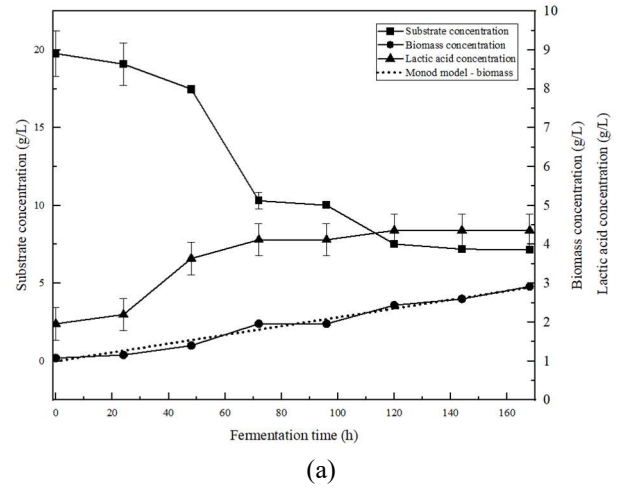


(c)

Fig. 3. Effect of agitation rate at 100 rpm on batch fermentation with *Bifidobacterium bifidum* TISTR 2129. (a) Monod model, (b) Logistic model and (c) Gompertz model.



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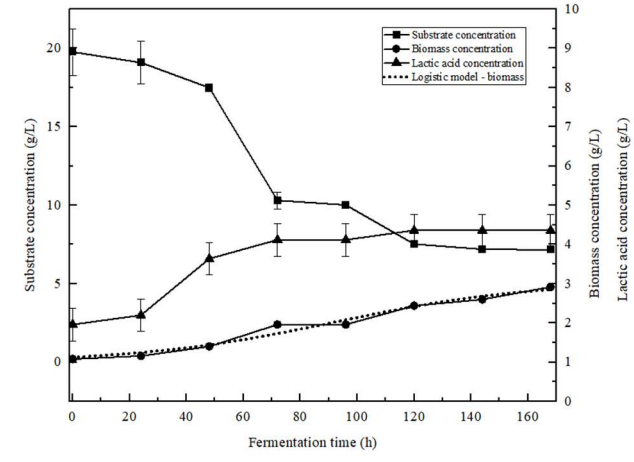

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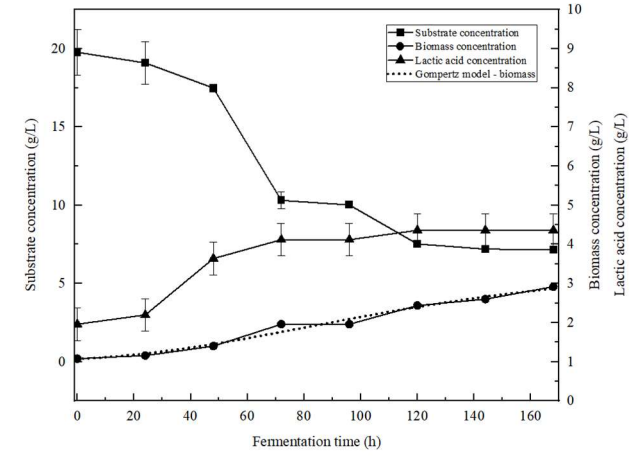

(c)

Fig. 4. Effect of agitation rate at 300 rpm on batch fermentation with *Bifidobacterium bifidum* TISTR 2129. (a) Monod model, (b) Logistic model and (c) Gompertz model.

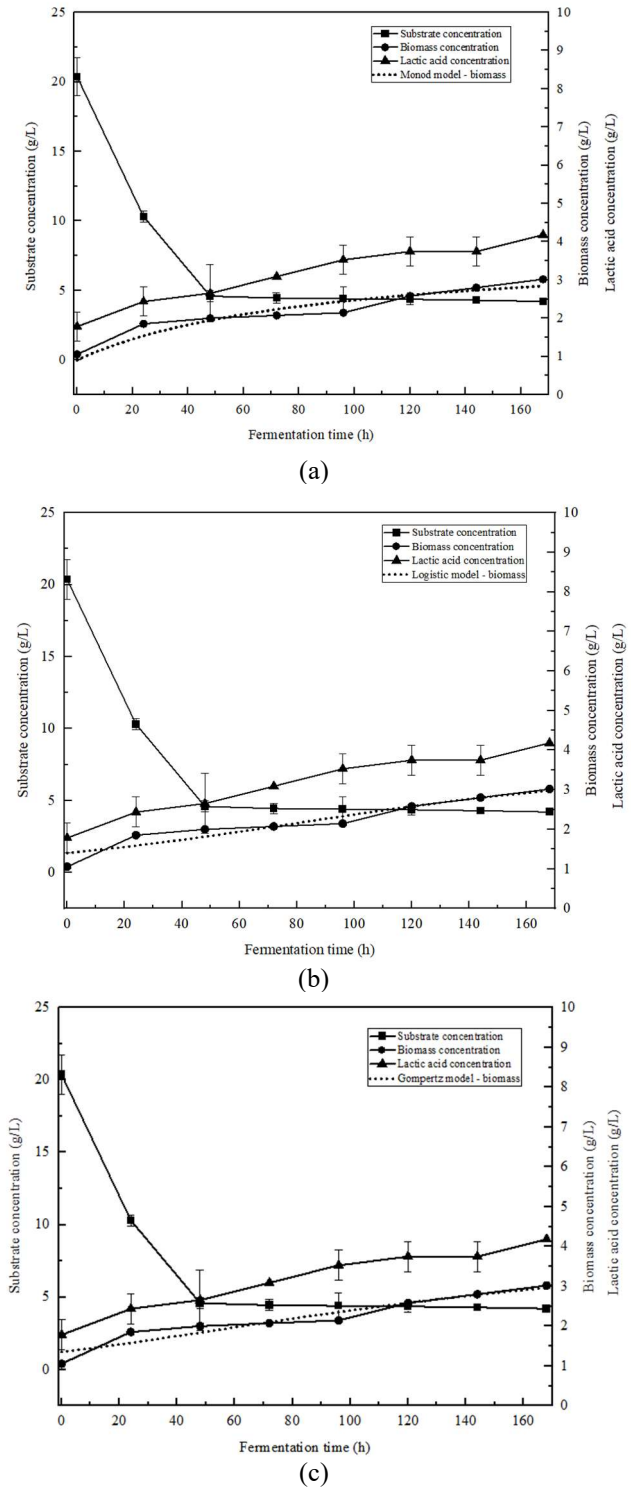

Fig. 5. Effect of agitation rate at 500 rpm on batch fermentation with *Bifidobacterium bifidum* TISTR 2129. (a) Monod model, (b) Logistic model and (c) Gompertz model.

3.3 Short-Chain Fatty Acid Production

The production of SCFAs by *B. bifidum* TISTR 2129 at different agitation rates (100, 300 and 500 rpm) is presented in Table 3. Lactic acid and acetic acid were produced at, in comparison, low concentrations, 52.21 mg/L and 33.15 mg/L, respectively, at an agitation rate of 100 rpm. Propionic acid was undetectable, and butyric acid was detected at a low concentration of 32.06 mg/L. Limited oxygen transport and nourishment mixing due to unsuitable agitation probably inhibited bacterial metabolism. Bifidobacteria, being anaerobic, grow in low-oxygen environments; yet insufficient substrate offer may have impeded their growth and short-chain fatty acid production (Macfarlane and Macfarlane, 2003). The production of SCFAs experienced a significant increase at 300 rpm, with lactic acid achieving the highest concentration (2711.73 mg/L), followed by acetic acid (618.77 mg/L), propionic acid (141.70 mg/L), and butyric acid (710.16 mg/L). Moderate agitation promotes the distribution of oxygen and nutrients, allowing proper fermentation. Bifidobacteria primarily metabolize carbohydrates through the fructose-6-phosphate phosphoketolase pathway, increasing the formation of lactic and acetic acids (Usta-Gorgun and Yilmaz-Ersan, 2020; De Bruyn et al., 2024). The promoted butyric acid indicates cross-feeding or metabolic flexibility in optimal conditions. However, the concentration of lactic acid experienced a significant decrease to 8.16 mg/L when the agitation rate was increased to 500 rpm. Butyric acid production remained comparatively high at 617.42 mg/L, while acetic acid and propionic acid were produced at lower concentrations than at 300 rpm. Previous studies found that *B. bifidum* DSM 20239 produces lactic, acetic, propionic, and butyric acids during fermentation. This indicates that salep was a readily fermentable substrate for this strain. It was important to point out that the growth of bifidobacteria was significantly facilitated by the addition of 1% (w/v) salep, which was comparable to the growth of 1% (w/v) glucose, a carbon source that was regularly used (Usta-Gorgun and Yilmaz-Ersan, 2020).

3.4 Kinetic Parameters

The kinetic parameters from Table 4 demonstrate that increasing agitation rates positively influence the growth and metabolic activity of *B. bifidum* up to a point. As agitation increases from 100 to 300 rpm, both biomass formation rate (r_x) and lactic acid production rate (r_p) significantly improve, reflecting enhanced substrate uptake (r_s) and more efficient conversion of glucose into biomass and product. However, while the highest agitation at 500 rpm further increases substrate consumption, the specific substrate uptake (q_s) per cell rises sharply, indicating lower substrate-use efficiency. At 500 rpm, the maximum biomass formation rate (r_x= 0.0321 g-cell/L-h) and lactic acid production rate (r_p= 0.0393 g/L-h) were achieved, indicating that higher agitation improved mass transfer and promoted more rapid microbial activity. However, the corresponding specific glucose consumption rate (q_s = -0.0228 g-glucose/g-cell-h) was markedly higher at 500 rpm, suggesting less efficient substrate utilization compared to

300 rpm. Similarly, although lactic acid production per cell (q_p) peaks at 300 rpm, it slightly decreases at 500 rpm, suggesting that excessive agitation may induce stress, such as oxidative damage or shear forces, reducing cellular metabolic efficiency. Interestingly, moderate agitation (300 rpm) provided a balance between growth and metabolic efficiency, showing an elevated specific biomass formation rate (q_x = 0.0057 g-cell/g-cell-h) and the highest specific lactic acid production rate (q_p = 0.0074 g-lactic-acid/g-cell-h). In contrast, fermentation at 100 rpm resulted in the lowest rates across all parameters, reflecting limited nutrient transfer under reduced mixing intensity. The higher substrate consumption at 500 rpm may reflect increased maintenance metabolism or stress responses, not proportional cell growth, due to possible shear stress. Overall, 300 rpm appears to strike an optimal balance between maximizing growth and product formation while maintaining metabolic efficiency, highlighting the importance of controlling agitation to support the microaerotolerant nature of *B. bifidum* during fermentation. These findings highlight that while high agitation accelerates biomass and metabolite accumulation, moderate agitation optimizes metabolic efficiency, offering critical insights for scaling up probiotic fermentations.

3.5 Kinetic Modeling and Parameter Estimation

The kinetic modeling of *B.bifidum* growth under different agitation rates (100, 300, and 500 rpm) provides insights into how varying oxygen transfer and mixing conditions influence microbial behavior in a fermentation environment. Among the three models applied Monod, Logistic, and Gompertz, the Gompertz model delivered the best fit to the experimental data as shown in Fig. 2–4 and tabulated values in Table 5, particularly at 300 rpm where the coefficient of determination (R^2) reached 0.98. This indicates that the Gompertz model effectively captured the full microbial growth profile, including the lag, exponential, and stationary phases. The flexibility of this sigmoidal model allowed it to reflect subtle biological transitions, such as the acceleration of growth at moderate agitation and potential inhibition at higher agitation rate (500 rpm), where μ_{max} declined slightly.

The Logistic model also performed well, producing consistent μ_{max} values (0.03 h^{-1} at 100 and 300 rpm;0.02 h^{-1} at 500 rpm) with R^2 values consistently above 0.90. This model accounted for environmental limitations, such as nutrient depletion or the impact of accumulated metabolic byproducts, which are particularly relevant in batch culture systems. Although both sigmoidal models (Gompertz and Logistic) provided good fits, the Gompertz model's superior R^2 and capacity to describe asymmetric growth phases made it more suitable for characterizing the complex dynamics of *B. bifidum*. These models do not explicitly account for substrate depletion and thus may overestimate growth potential in late stages. In contrast, the Monod model, which traditionally relates microbial growth to substrate concentration, showed limitations in this study. While it yielded reasonable parameter estimates at 100 rpm, at 300 rpm the values for μ_{max} (3.49 × 1014 h^{-1}) and K_s (1.24 × 10^{16} g/L) were unreasonably high, suggesting overfitting and poor biological relevance despite a seemingly good R^2 (0.97). The kinetic parameters presented in Table 5 demonstrate the performance of three microbial growth models at varying agitation rates (100, 300, and 500 rpm) for *B. bifidum* TISTR 2129. The Monod model showed high maximum specific growth rates (μ_{max}) at higher agitation rates (e.g., 8.11 at 500 rpm), but its fitting was less accurate at 100 rpm with an R^2 value of 0.77 and produced unrealistically large K_s values at 300 rpm and 500 rpm, suggesting overfitting and limited applicability. In contrast, the Logistic and Gompertz models both provided better fits with higher R^2 values (0.97 and 0.98), particularly the Gompertz model, which excelled in capturing the lag phase, exponential growth, and stationary phase, making it the most suitable model for describing the microbial growth dynamics in this study. The Gompertz model's ability to describe asymmetric growth, including the lag and stationary phases, provided a more accurate representation of microbial behaviour, especially at 300 rpm, where the highest fit was observed. This suggests that while the Monod model is useful for substrate-limited growth, the Gompertz model is preferred for microbial systems exhibiting more complex growth patterns.

This mismatch indicates that the Monod model is not appropriate for describing *B.bifidum* growth under moderate to high oxygen transfer, likely because it does not account for factors such as oxygen inhibition or saturation effects that are especially important for microaerotolerant organisms. Overall, the results show that increasing agitation improves substrate uptake and promotes cell growth up to a point, with 300 rpm identified as the optimal condition. This rate enhances glucose utilization and biomass production without causing significant oxidative stress, as evidenced by improved growth kinetics and lactic acid production. However, further increases in agitation (500 rpm) appear to introduce mild stress, reflected in slightly lower μ_{max} values and model fits. As shown in Fig.3, the turbidity at the agitation rate of 500 rpm is lower than at 300 rpm, which could be due to the excessively high agitation rate inhibiting the growth of the microorganisms. The lighter turbidity at 500 rpm after extended fermentation may result from cell lysis or reduce viable biomass, consistent with potential agitation-induced stress.

In this study, three commonly used microbial growth models (the Monod, Logistic, and Gompertz models) were applied to describe the growth kinetics of *B. bifidum* TISTR 2129 under varying agitation rates (100, 300, and 500 rpm) in a stirred-tank bioreactor. The Gompertz model was the most accurate and reliable for describing the growth kinetics of *B. bifidum* in this study. Its ability to account for the lag phase, exponential growth, and stationary phase, along with its flexibility in capturing complex growth behaviours under

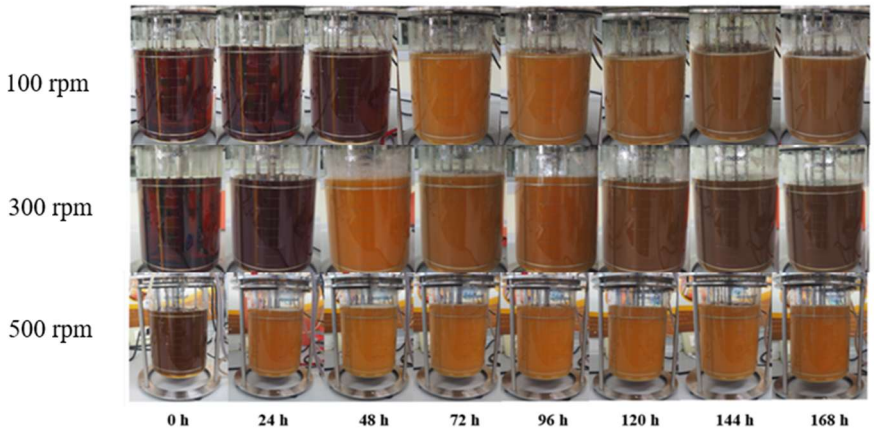

Fig. 6. Visual representation of fermentation broth of *Bifidobacterium bifidum* TISTR 2129 at different agitation rate over a 168-h incubation period at 37°C.

Table 3. SCFAs production

Agitation rate (rpm)	SCFAs (mg/L)			
	Lactic acid	Acetic acid	Propionic acid	Butyric acid
100	52.21	33.15	-	32.06
300	2711.73	618.77	141.70	710.16
500	8.16	42.11	95.05	617.42

Table 4. Kinetic parameters associated with probiotic growth.

Agitation rate (rpm)	r_x (g-cell/L-h)	r_s (g-glucose/L-h)	r_p (g-Lactic acid/L-h)	q_x (g-cell /g-cell-h)	q_s (g-glucose /g-cell-h)	q_p (g-Lactic acid/g-cell-h)
100	0.0119	-0.0610	0.0143	0.0043	-0.0064	0.0051
300	0.0274	-0.0751	0.0357	0.0057	-0.0105	0.0074
500	0.0321	-0.0962	0.0393	0.0055	-0.0228	0.0068

Table 5. Kinetic parameter for microbial growth at 100, 300 and 500 rpm.

Model	μ_{max}			K_s			R^2		
	100rpm	300 rpm	500 rpm	100 rpm	300 rpm	500 rpm	100 rpm	300 rpm	500 rpm
Monod	3.49	3.49×10^{14}	8.11	55.14	1.24×10^{16}	88.43	0.77	0.97	0.90
Logistic	0.03	0.03	0.02	-	-	-	0.94	0.97	0.90
Gompertz	0.02	0.02	0.01				0.94	0.98	0.91

varying fermentation conditions, makes it the preferred model for kinetic analysis in this system. Therefore, the Gompertz model is recommended for modeling *B. bifidum* growth kinetics under microaerophilic environnements, and the findings reinforce the importance of maximizing agitation to balance mass transfer and oxygen sensitivity in probiotic fermentation systems.

This study indicated that agitation rate had a substantial influence on biomass growth, lactic acid production, and SCFAs synthesis by *B. bifidum* TISTR 2129 in a stirred-tank bioreactor. Among the tested conditions, agitation at 500 rpm resulted in the highest biomass (5.8 g/L) and lactic acid concentration (9.0 g/L) after 168 h of fermentation, indicating enhanced microbial activity and improved substrate utilization. However, when comparing these findings with studies that employed shorter fermentation periods (e.g., 72 h or 120 h), there was a noticeable difference in the overall yield and productivity rates. For instance, previous research has shown that shorter fermentation durations often result in suboptimal yields of SCFAs and biomass due to the insufficient time for the microorganisms to reach their maximum growth phase and metabolite production (Usta-Gorgun and Yilmaz-Ersan, 2020). On the other hand, studies using 72-h fermentations generally report lower metabolic yields, particularly for SCFAs, suggesting that shorter fermentation may not allow adequate accumulation of these bioactive compounds (De Bruyn et al., 2024). Increased agitation facilitated mass and

nutrient transfer, which in turn supported bacterial proliferation and lactic acid synthesis. However, maintaining an optimal balance proved essential, as excessive agitation appeared to reduce SCFAs diversity, especially lactic acid output. SCFAs analysis further revealed that moderate agitation (300 rpm) promoted broader metabolic diversity and higher yields of key metabolites, including lactic acid (2711.73 mg/L), acetic acid, propionic acid, and butyric acid. At 500 rpm, enhanced sugar uptake favored biomass accumulation and glycolysis toward lactic acid early in fermentation, but by 168 h, lactic acid was further metabolized or diverted to other pathways, resulting in low residual SCFAs. While higher agitation boosted overall growth and lactic acid output, moderate agitation supported a wider range of SCFAs production, likely due to improved metabolic regulation under mildly anaerobic conditions. While 168 h of fermentation yields high quantities of metabolites and biomass, it is important to consider the cost-effectiveness of prolonged fermentations. Longer fermentation periods may increase operational costs, energy consumption, and labor costs, making them less feasible for large-scale industrial applications where time and cost efficiency are critical. In this regard, the optimal fermentation duration for maximizing yields must be balanced with production costs. A more time efficient fermentation process using, for example, 120-h or 144-h fermentations, may provide similar yields in some cases, but with reduced costs and faster turnover times. Therefore, while this study demonstrates that 168 h of fermentation is effective for maximizing yields, further research is needed to explore optimal fermentation durations that strike a balance between high yield and cost-effectiveness, with attention to scale-up potential for industrial fermentation processes.

4. CONCLUSION

Agitation rate plays a critical role in shaping the growth, metabolite production, and SCFAs profile of *B. bifidum* TISTR 2129 in stirred-tank bioreactors. High agitation (500 rpm) maximizes biomass and lactic acid production, whereas moderate agitation (300 rpm) promotes greater SCFAs diversity and metabolic balance. While prolonged fermentation (168 h) ensures high yields, shorter durations may offer comparable results with improved cost-effectiveness and operational efficiency. These findings highlight the importance of optimizing both agitation and fermentation time to achieve a balance between maximum yield, metabolic diversity, and industrial feasibility for probiotic production.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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