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Production of L-tryptophan for food, feed, and pharmaceutical applications

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ABSTRACT

Background: L-tryptophan can be utilized in a variety of applications. The prevalence of L-tryptophan in diverse sectors, including chemical, pharmaceutical, food and feed industries, underscores its significance. The predominant method of producing L-tryptophan involves microbial fermentation, which requires establishing an effective culture of microorganisms.

Objective: This study focuses on *Brevibacterium flavum* C18 characterization and the technological parameter optimization for the biotechnological deriving of L-tryptophan.

Methods: m-Fluorophenylalanine- and 5-fluorotryptophan-resistant C18 (m-FP^r, 5-FT^r) were characterized by a range of morphological and biochemical properties. A microbiological assay evaluated the potential of this strain to synthesize L-tryptophan. The effect of antibiotics on the strain was determined by agar disk diffusion assay. Finally, the fermentation was optimized by varying the agitation speed of the incubator shaker during shake-flask fermentation.

Results: C18 was found to be non-motile, gram-positive, and prototrophic. This strain was synthesized from L-tryptophan through the utilization of microbiological testing methodologies.

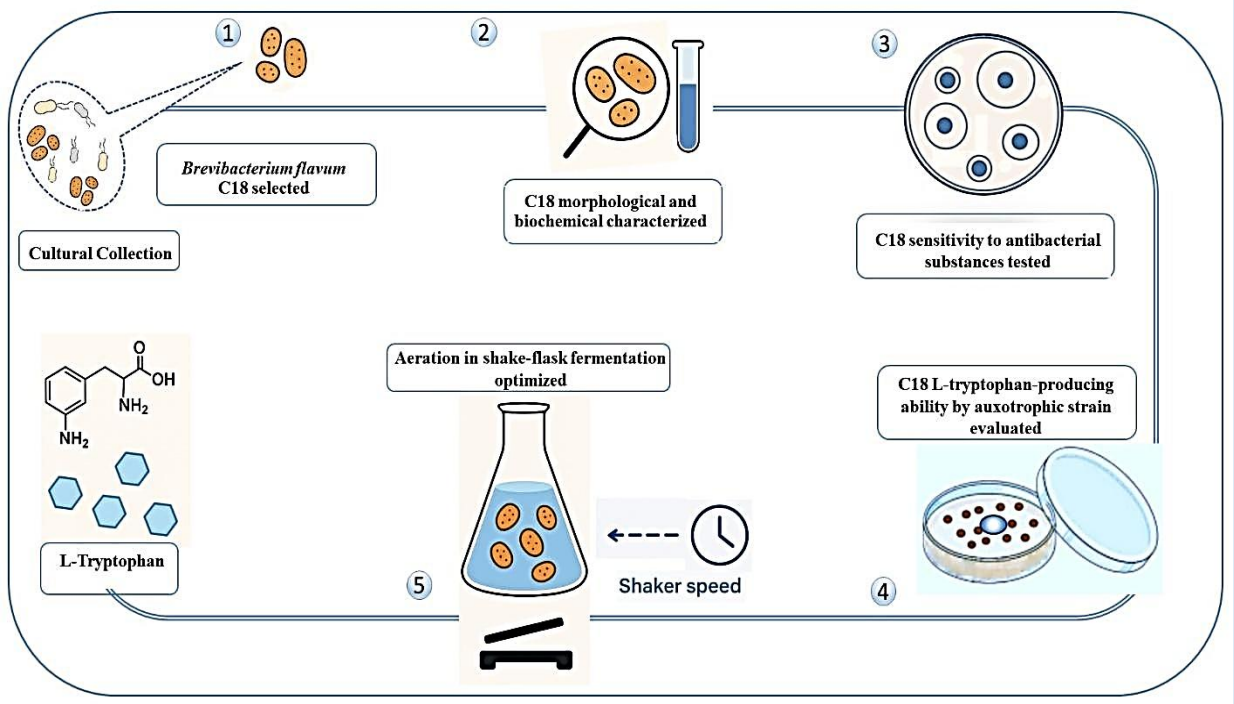
Conversely, C18 exhibited a positive response in the catalase test, as indicated by the citrate test, gelatin, and starch liquefaction tests. The strain demonstrated sensitivity to kanamycin, tetracycline, and vancomycin. Furthermore, the strain demonstrated heightened sensitivity to gentamicin and erythromycin.

The highest yield of L-tryptophan (up to 7.8 g/l) by C18 was achieved at a stirring rate of 220 rpm in shake-flask fermentation.

This research allowed assessing the impact of agitation speed on L-tryptophan production using producer C18. The study indicates that, compared to the described strains, L-tryptophan production has been enhanced by a safe producer that is more resilient and adaptable to environmental conditions.

Conclusion: The investigation has demonstrated that C18 is characterized by a combination of morphological and biochemical properties. Furthermore, it was determined that the synthesis of L-tryptophan was optimized by agitation speed, with the maximum amount of the target amino acid being recorded at a speed of 220 rpm. The subject strain may hold significant promise in terms of its potential for application in the nutritional and pharmaceutical sectors.

Keywords: *Brevibacterium flavum*, morphological and biochemical characteristics, agitation speed, L-tryptophan production, bioactive compound.



INTRODUCTION

Tryptophan is distinguished by its unique indole ring structure [1]. It is imperative for the functioning of numerous organs within the body [2]. It has been demonstrated to regulate the synthesis of bioactive compounds, including protein, vitamin B3, serotonin,

melatonin, and niacin, among others [3-5]. This amino acid plays a pivotal role in regulating appetite, sleep, mood, and pain levels, among other physiological functions. Tryptophan takes part in the synthesis and converts to proteins or peptides and is used by the body, where it is converted to bioactive compounds. The degradation process is primarily facilitated by two parallel metabolic pathways: the 5-hydroxytryptamine (5-HT) and the kynurenine ways. These pathways under consideration will synthesize some secondary compounds. Serotonin compounds encompass 5-hydroxytryptophan (5-HTP) as well as serotonin and melatonin. The kynurenine metabolic pathway encompasses kynurenine and niacin [67]. The final products of these pathways are melatonin and niacin [1]. Consequently, it plays a pivotal role in human metabolism, growth, and development [8-9].

L-tryptophan is not produced by the body and must therefore be supplied through dietary intake. As is well established in the relevant literature, the substance is found in red meat, poultry, fish, eggs, and dairy products [10]. The daily doses of amino acids recommended for adults range from 250 mg to 425 mg, which equates to 3.5-6 mg per kilogram of body weight. Newborns and children require significantly higher levels of tryptophan in their diet than adults do, approximately 12 mg per kilogram of body weight per day. The nutritional and medicinal values of L-tryptophan have led to its extensive utilization within the pharmaceutical industry, where it is employed as a sedative and a mild antidepressant. Additionally, it has found application in the food industry as a food and feed additive. The substance is produced in its pure amino acid form as a bioactive supplement and in various amino acid complexes [7-8].

According to QYResearch, the global L-tryptophan market is projected to attain a valuation of $1915 million till 2030, exhibiting a product yearly increase of 13.7% (2024-2030) [11].

In many cases, L-tryptophan is synthesized chemically, by direct fermentation, or enzymatically. However, with the advent and subsequent expansion of the green business sector, demand for chemical synthesis has declined. Enzymatic conversion is an industrially viable method for producing amino acids; however, it is a costly process. In sustainable chemical synthesis, microbial fermentation is often preferred over chemical synthesis and enzymatic conversion. This is because it enables the eco-friendly synthesis of L-tryptophan from low-cost and renewable carbon sources [12].

L-tryptophan has been demonstrated to be capable of producing a wide variety of bacterial strains, including but not limited to *Brevibacterium*, *Corynebacterium*, *Escherichia* and *Bacillus*, amongst others. At present, *Corynebacterium glutamicum* and *Escherichia coli* are the predominant producers of L-tryptophan [13-15].

The metabolic pathway of tryptophan in coryneform bacteria is similar to that in *E. coli*, with some genetic differences. On the other hand, coryneform bacteria is generally known as safe [16].

An alternative method for enhancing L-tryptophan production is to cultivate more resilient and adapted fermentation strains to their environment [12].

The objective of the present investigation is twofold: firstly, to characterize L-tryptophan-producer *Brevibacterium flavum* C18, and secondly, to optimize the technological parameters for L-tryptophan production by the biotechnological way.

MATERIALS AND METHODS

Strain: The L-tryptophan-producer that was the subject of the present study was *B. flavum* C18. The strain was found to be resistant to m-fluorophenylalanine (m-FP^r) and 5-fluorotryptophan (5-FT^r).

In this study, a range of strains from the Culture Collection were examined, including a positive control, Azotobacter vinelandii AV1 (GenBank accession number:

MK847515.1), and a negative control, Rhizobium pusense RP1 (GenBank accession number: MT670424.1), for biochemical tests. The strain RP1 was a positive control for the microbiological test.

A wild-type prototrophic strain *B. flavum* ATCC 14067, an L-alanine producer *B. flavum* AA5 (DL-α-ABA^r, D-ala$^-$), a L-valine producer *B. flavum* V12 (DL-α-ABA^r, HA^r, ile$^-$), and an indicator strain *B. flavum* trp$^-$ (trp$^-$), were analyzed for a phenotypic confirmation of the L-tryptophan-producing strain.

Media: Nutrient agar (%): Animal tissue peptic digest – 1.0, extract of meat – 1.0, NaCl – 0.5, agar – 1.5, pH: 7.6.

Nutrient broth (%): Animal tissue peptic digest – 1.0, extract of meat – 1.0, NaCl – 0.5, pH: 7.6.

Simmon's citrate agar (%): NaCl – 0.5, $Na_3C_6H_5O$ – 0.2, $(NH_4)H_2PO_4$ – 0.1, K_2HPO_4 – 0.1, bromothymol blue – 0.008, agar – 1.5, pH: 7.0.

Starch agar (%): Starch – 0.2, agar – 1.5, nutrient broth – up to 1 L, pH: 7.4.

Gelatine agar (%): Gelatin – 10.0, agar – 1.1, nutrient broth – up to 1 L, pH: 7.4.

Tryptose agar (%): Tryptose – 1.0, NaCl – 0.5, agar – 0.5, pH: 7.4.

Minimal agar (%): Glucose – 1.0, NH_4Cl – 0.5, NH_4NO_3 – 0.1, Na_2SO_4 – 0.2, K_2HPO_4 – 0.3, $MgSO_4 \cdot 7H_2O$ – 0.025, KH_2PO_4 – 0.1, $FeSO_4 \cdot 7H_2O$ – 0.0001, $MnSO_4 \cdot 5H_2O$ – 0.0001, biotin – 0.00004, thiamine chloride – 0.00004, agar – 1.5 or with L-tryptophan – 0.00002, pH: 7.4.

Fermentation medium (%): sucrose – 15.0, $(NH_4)_2SO_4$ – 5.5, KH_2PO_4 – 0.01, $MgSO_4$ – 0.01, $CaCO_3$ – 5.0, $FeSO_4 \cdot 7H_2O$ – 0.0001, $MnSO_4 \cdot 5H_2O$ – 0.0001, biotin – 0.00004, thiamine chloride – 0.00004, pH: 7.4.

Morphological and biochemical characteristics: The following morphological properties were tested for the strain: Gram stain, shape, size, and motility of cells. A series of biochemical tests was conducted to characterize the L-tryptophan-producing strain. The principal strategy employed for this purpose is the implementation of a catalase test, a gelatin hydrolysis test, a starch hydrolysis test, and a citrate utilization test.

Morphological characterization of the cells of the L-tryptophan producer was conducted using a microscope Leica DM500 [17-18].

For the cells' motility test, tryptose agar was utilized. The movement was observed in a diffuse zone of growth extending from the line of inoculation.

To perform the citrate utilization test, the Simons citrate agar slants were first inoculated with the L-tryptophan producer and incubated at 30°C (Daihan Scientific Wisd WiseCube WIG incubator) for 72 h. It was noted that the positive slants changed color from green to blue.

The catalase test was conducted by adding a small quantity of the strain from a nutrient agar slant to 3% hydrogen peroxide. The presence of oxygen bubbles indicated a positive catalase result.

To carry out the gelatin hydrolysis test, the gelatin agar was first inoculated by the L-tryptophan producer and then kept at 30°C for 72 h. The presence of light zones around gelatinase-positive cultures on the plate indicates gelatin hydrolysis.

The starch agar was inoculated with the L-tryptophan producer and incubated at 30°C for 48 h before being flooded with Lugol's iodine solution. The presence of light zones on all sides of the amylase-positive culture indicates starch hydrolysis on the plate.

Effect of antibacterial substances: Antibacterial activity was tested by the disc diffusion method. The tests were conducted using five different antibiotic discs (30 µg/disc vancomycin, 30 µg/disc kanamycin, 10 µg/disc

gentamicin, 15 µg/disc erythromycin, 30 µg/disc tetracycline). The antibiotic discs were placed on a nutrient agar plate containing the strain and incubated at 30°C for 5 days. Antibiotic effect was determined by measuring the diameter of the light zone.

Inoculum: An inoculum was conducted using the L-tryptophan-producing strain in nutrient broth at 30 °C, 220 rpm (Biosan ES-20/80C shaker, orbital diameter: 2.0 cm) for 18h.

Shake-flask fermentation: The shake-flask fermentation was performed in an Erlenmeyer flask with 1 mL inoculum (2.5 OD_{540}) and 15 mL fermentation medium. The shake-flask fermentations were carried out at 30 °C, 100-250 rpm (New Brunswick Scientific Innova 43 shaker, orbital diameter: 2.5 cm), for 96 h and an initial pH of 7.0 (Biobase 210 Benchtop pH meter).

L-tryptophan-producing strain growth: L-Tryptophan-producer growth was quantified by the optical density (Drawell DU-8200 Single Beam UV/VIS spectrophotometer).

L-tryptophan qualitative determination: L-tryptophan qualitative determination was performed by means of microbiological assay using L-tryptophan auxotrophic strain trp^-.

Initially, the phenotypic confirmation of prototrophic *B. flavum* C18 and the L-tryptophan auxotrophic strain trp^- was carried out. This was done alongside the wild-type strain 14067 and the auxotrophic strains AA5 and V12 as control**s**. The wild-type strain 14067 served as a positive control. The D-alanine auxotrophic strain AA5 and the L-isoleucine auxotrophic strain V12 served as negative controls.

This was done by streaking the cultures on minimal agar. This agar was supplemented with and without L-tryptophan. The dish was kept at 30°C for 5 days. After incubation, the growth of the strains was observed (i.e. the nutrients required for the growth of the auxotrophic cultures) and compared with that of the control strains.

In the subsequent experiment, for the identification of *B. flavum* C18 L-tryptophan synthesizing potential by spreading a dominant number of indicators, the L-tryptophan-auxotrophic strain trp- (107 colony forming unit (CFU)/mL (Funke Gerber ColonyStar colony counter)) was mixed with the C18 strain on the minimal agar plate. The dishes were kept at 30°C for 5 days.

L-tryptophan quantitative determination: The quantitative determination of L-tryptophan was done using the descending paper chromatography method [19].

RESULTS AND DISCUSSION

Morphological and biochemical characterization of L-tryptophan producer: It was shown that *B. flavum* C18 was Gram-positive with rod-shaped cells, with an average cell size of 0.4 × 0.3 µm (L × W) after 48 h growth on nutrient agar. Cells were typically arranged singly or in pairs and were non-motile (Figure 1). It was catalase-positive, but could not hydrolyze starch, gelatin, and utilize citrate as a source of energy (Figure 2). Other authors reported a similar result: the strains of the genus *Brevibacterium* were Gram-positive, non-spore-forming, catalase-positive bacteria [20, 21].

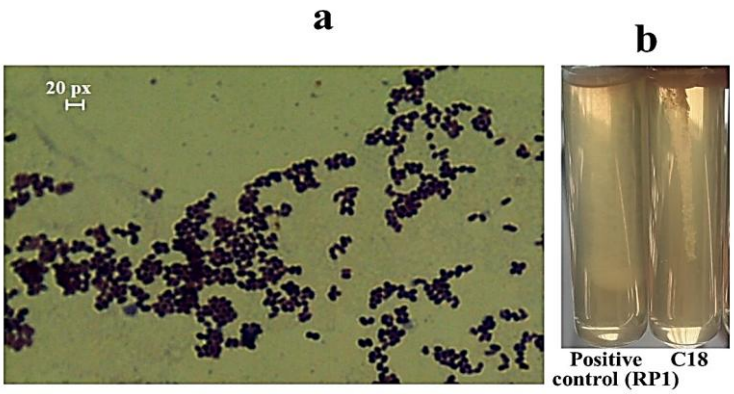


Figure 1. Morphological some characteristics of L-tryptophan producer. *a – micrograph of B. flavum C18 (× 10 000; 1 px = 263.6 µm); b – motility test.*

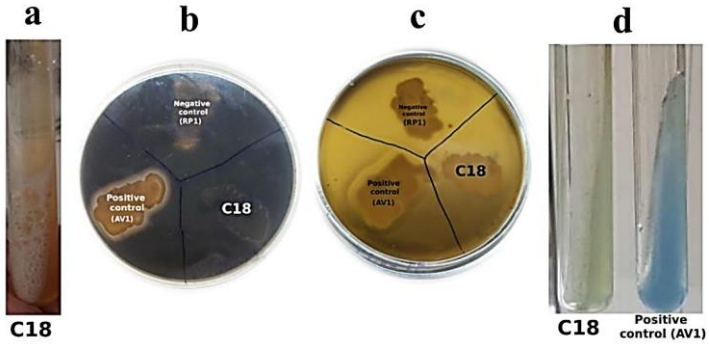


Figure 2. Biochemical characteristics of L-tryptophan producer. *a – catalase test; b – starch hydrolysis test; c – gelatin hydrolysis test; d – citrate utilization test.*

Antibacterial substances' effect on L-tryptophan producer: The issue of microbial resistance to antibacterial substances is becoming increasingly pointed each year as the spread of resistance in microbial communities has an accumulative nature. Specific features of bacteria facilitate the transfer of movable genes of antibiotic resistance. These characteristics play a pivotal role in contaminating novel microbial communities and displacing bacteria that are less able to withstand antibiotics [22].

In the present study, it was demonstrated that C18 formed clear zones of inhibition with different degrees of intensity around discs of kanamycin, vancomycin, tetracycline, gentamicin and erythromycin. These zones were considered response to the antibiotics mentioned above (Figure 3, Table 1).

Table 1. L-tryptophan producer response to antibiotics.

Antibiotic	C18
	Diameter of inhibition zone, mm
gentamicin	36
erythromycin	40
vancomycin	26
kanamycin	28
tetracycline	18

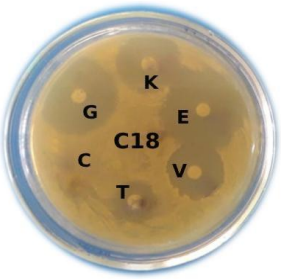


Figure 3. Antibiotic feedback from L-tryptophan producer. *C – control (distilled water); K – kanamycin;G – gentamicin; E – erythromycin; T – tetracycline; V – vancomycin .*

The antibacterial substances response profile demonstrated that the most active antibiotic was erythromycin, followed by gentamicin, kanamycin, vancomycin, and tetracycline. *Brevibacterium paicivorans* were susceptible to gentamicin, vancomycin, etc. [23]. Another strain, Brevibacterium ravenspurgense, was susceptible to gentamicin, vancomycin, and tetracycline but resistant to erythromycin [20]. In contrast, *Brevibacterium* spp. was low-level resistant to vancomycin, tetracycline, etc. [21].

L-tryptophan production by microbiological mean: The L-tryptophan producer C18 demonstrated growth in minimal medium, both in the absence (Figure 4a) and the presence (Figure 4b) of L-tryptophan. Notably, it maintained its prototrophic phenotype (Table 2).

Conversely, the indicator strain *B. flavum* trp$^-$ exhibited no growth in minimal medium devoid of L-tryptophan. The strain has demonstrated satisfactory growth when cultivated on minimal medium with L-tryptophan (Figure 4b). The results indicate that the indicator strain exhibited an auxotrophic phenotype, which is a prerequisite for its growth.

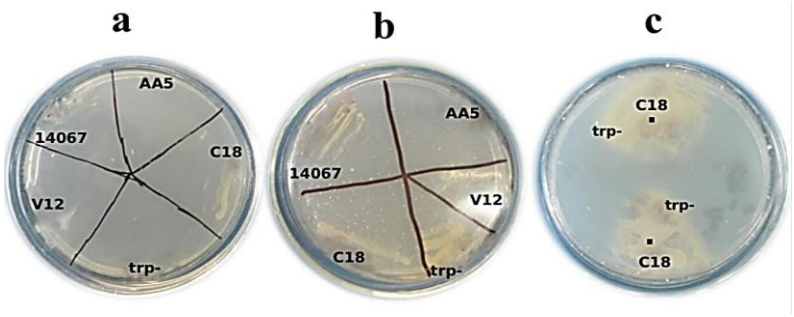


Figure 4. Growth of *B. flavum* strains on minimal media. *a – medium without L-tryptophan; b – medium with L-tryptophan; c – medium without L-tryptophan and with indicator strain.*

Table 2. Phenotypic conformation of strains *B. flavum*.

Strain	Minimal medium without L-tryptophan	Minimal medium with L-tryptophan
positive control (14067)	+	+
negative control (AA5)	-	-
negative control (V12)	-	-
C18	+	+
trp$^-$	-	+

Notes: '+' – growth; '-' – no growth.

In accordance with the prevailing growth status (Figure 4c), the co-culture with the L-tryptophan producer resulted in the formation of substantial colonies, which were accurately determined. A similar relationship was found in case of the indicator strain and other L-tryptophan-producing *B. flavum* strains, resistant to m-fluorophenylalanine (m-FP^r) or p-fluorophenylalanine (p-FP^r) [13].

Enhancement of L-tryptophan production in B. flavum C18: The growth of bacteria and the subsequent production of their metabolic by-products are significantly influenced by the composition of the medium and various physical factors, including agitation speed, etc. It is crucial to recognize the importance of both agitation and aeration as pivotal parameters in aerobic processes. During fermentation, agitation plays a pivotal role in the mixing stages. The apparatus has been demonstrated to enhance mass and oxygen transfer between the phases, whilst also maintaining homogeneous chemical and physical conditions in the medium through continuous mixing. Conversely, agitation has been shown to induce shear forces, which in turn exert a significant influence on microorganisms. This influence can be observed in a variety of ways, including alterations in morphology, variations in growth patterns, and changes in metabolite formation. Aeration is a pivotal process in the fermentation of products, enhancing the rate of oxygenation. Mixing the fermentation medium is also important to the process, particularly when mechanical agitation is low. Product formation could be increased by optimizing the agitation or aeration rates [11, 16].

Agitation speeds affect tryptophan biosynthesis and carbon source consumption. Metabolic reactions deplete available oxygen; a high oxygen demand indicates accelerated growth and high carbon source consumption. As a carbon source is depleted, metabolism is reduced, increasing agitation speed; therefore, carbon source availability and growth rate can be indirectly controlled by maintaining agitation speed [16].

The present study investigates the impact of agitation on L-tryptophan production by C18 (m-FP^r, 5-FT^r) and its growth. To this end, a comparison was made of the shaker performance at four agitation speeds: 100, 150, 220, and 250 rpm (Figure 5).

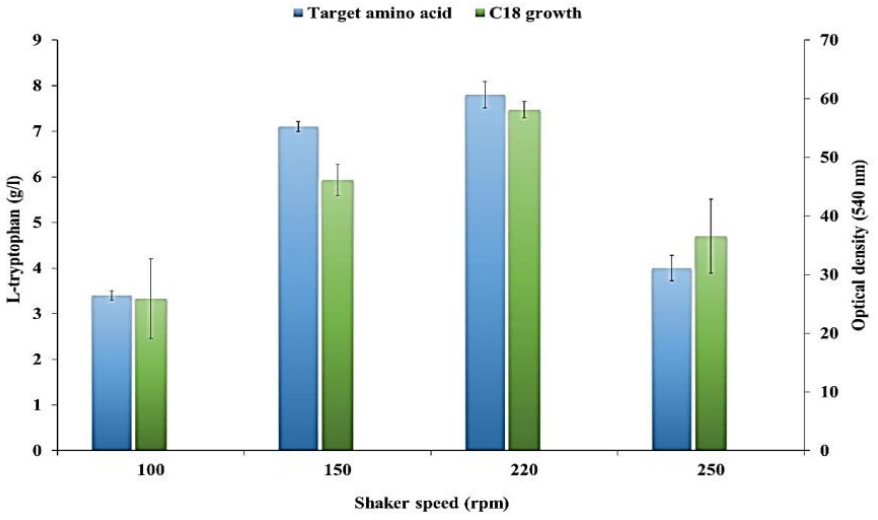


Figure 5. Effect of agitation speed on L-tryptophan production (mean ± standard deviation, n = 2).

The maximum final production of L-tryptophan and growth of the producer was achieved at 220 rpm in 96 h (7.8 g/l and 58.1 OD540), followed by 150, 250, and 100 rpm. At the optimal agitation rate (220 rpm), *B. flavum* G27 and strain 59C (m-FP^r, 5-FT^r) demonstrated the capacity to synthesize up to 6 g/l of L-tryptophan [8]. *B. flavum* 4 (m-FP^r) was found to be capable of producing up to 3.4 g/l target amino acid in the same conditions [24]. As demonstrated in [11], the maximum production of L-tryptophan by other producers (strain 18 (m-FP^r) and strain 27 (p-FP^r), from the same species) was found to be 2.0 g/l. In contrast, a genetically engineered coryneform bacterium operating under similar conditions produced 50.5 g/L L-tryptophan within 48 h [25]. The results obtained demonstrate that, in contrast to the effects observed in known strains [11, 22], the introduction of additional resistance to the amino acid structural analogue, in conjunction with the optimization of agitation speed for L-tryptophan production, resulted in the growth of the producer and an enhancement in its productivity. This study provides findings on the role of agitation speed in amino acid fermentation, emphasizing its significance for L-tryptophan production via green biosynthesis. Nevertheless, a more thorough investigation is necessary across fermentation conditions to formulate a resolution concerning the potential of agitation to affect L-tryptophan production and producer growth. The findings lend support to the hypothesis that the producer under discussion, in its capacity as a safe fermentation strain, may hold significant promise for application in the nutritional and pharmaceutical industries.

CONCLUSIONS

The present study has demonstrated that *B. flavum* C18, selected from the culture collection for its resistance to various structural analogues (m-fluorophenylalanine and 5-fluorotryptophan) of aromatic amino acids, exhibits a combination of morphological and biochemical properties. Furthermore, the important role of agitation for attaining maximum productivity in processes was demonstrated, with a high yield of L-tryptophan recorded at 220 rpm. Continued research in fine-tuning fermentation of all parameters will further improve the economic viability and scalability of L-tryptophan production.

List of Abbreviation: m-FP^r: m-fluorophenylalanine-resistant, 5-FT^r: 5-fluorotryptophan-resistant, 5-HT: 5-hydroxytryptamine, 5-HTP: 5-hydroxytryptophan, CFU: colony forming unit

Competing interests: The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Authors' contributions: GA: Project administration, supervision, investigation, methodology, writing – review and editing. LsM: Investigation, methodology, data curation, writing – original draft preparation, writing – review and editing. SK, GTs, SM, ZhK, LzM Investigation, methodology. AT and VGh: Investigation, writing, review, and editing.

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