ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Short Communication / JAPR / Sec. E
Salama et al., 2024, 8 (4), 231-237

Journal of Advanced Pharmacy Research

Section E: Microbiology & Immunology

Indole Production Capacity in Three Different *Escherichia coli* Strains

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Submitted on: 21-07-2024; Revised on: 15-08-2024; Accepted on: 23-08-2024

To cite this article: Salama, G. G.; El-Mahdy, T. S.; Moustafa, W. H.; Emara, M. Indole Production Capacity in Three Different *Escherichia coli* Strains. *J. Adv. Pharm. Res.* **2024**, *8* (4), 231-237. DOI: 10.21608/aprh.2024.306103.1283

ABSTRACT

Background: In *Escherichia coli*, the enzyme tryptophanase (TnaA) is responsible for the production of indole from tryptophan. However, the factors governing the production and excretion levels of indole by *Escherichia coli* remain uncertain, complicating the interpretation of experiments exploring the biological effects of indole at high concentrations. The objective of the study: Most investigations into indole signaling focused on concentrations within the range of 0.5-0.6 mM, like those found in the supernatant of *Escherichia coli* stationary phase. While reports on indole concentrations in human stool samples exhibit variability, the average concentration hovers around 2.6 mM. As a result, indole concentrations ranging from 0.5 to 1 mM are commonly considered physiologically relevant and are frequently used as experimental additives. **Methods**: We investigated the indole production capacity of three *Escherichia coli* strains under different conditions, including, medium composition, temperature, and pH to understand how these factors influenced the indole yield. **Results**: we demonstrated a direct relationship between tryptophan concentration in the growth medium and indole production, emphasizing the importance of this amino acid in metabolic pathways associated with indole synthesis. Moreover, we found that higher temperature and pH levels were linked to increased levels of indole production. **Conclusion**: This research underscores the intricate interplay between microbial metabolism and environmental cues influencing the indole production in three *Escherichia coli* strains. These insights are crucial for optimizing conditions in laboratory-scale experiments focused on indole production processes, particularly in understanding its impact as a signal molecule on biological activity at various levels of interspecies and interkingdom interactions.

Keywords: *Tryptophanase, E. coli O157:H7, Intestinal Microbiome, TnaB, Mtr*

INTRODUCTION

One of the most recognizable indole-producing bacteria is *Escherichia coli* (*E. coli*) which belongs to the *Enterobacteriaceae* family. It is a Gram-negative rod-shaped, non-sporulating, nonmotile or motile that can produce acid from glucose and reported to be catalase positive and oxidase negative. It is categorized among the first colonizing bacteria of the gut after birth and very competitive as a commensal bacterium being the most abundant among the human intestinal microbiota[1]. On the other hand, many pathogenic strains of *E. coli* like

serotype O157:H7, acquire specific virulence factors which enable them to cause a plethora of diseases including both intestinal and extraintestinal infections. Their facultative anaerobe nature is responsible for their excellent survival rates and ease of spreading to new hosts [2].

In *E. coli*, indole is generated by the cytoplasmic enzyme tryptophanase (TnaA), which reversibly hydrolyses tryptophan to create indole, pyruvate and ammonia, during a stationary cell growth phase, but no pathway for indole degradation is reported for this bacterium. It was concluded that bacteria synthesize tryptophan from indole as a carbon source although the equilibrium of the reaction favors the production of indole from tryptophan, and therefore indole test has been regularly used as a diagnostic marker for the identification of *E. coli*[3].

The TnaA protein in *E. coli* is responsible for catalyzing the process of indole production. It has been proposed that TnaA may play a role in the degradation of tryptophan synthesized by endogenous metabolic pathways. However, this potential action is thought to be counterproductive due to the potential inhibition of growth caused by excessive degradation of this amino acid[4]. While indole can diffuse across cell membranes without the need for a specific transporter, the import of tryptophan is regulated by TnaB. In the absence of TnaB, two other amino acid transporters, AroP and Mtr, can import sufficient tryptophan to support cell growth. The specific contribution of these importers to indole production remains unknown[5].

The amount of indole that bacteria can generate is a crucial matter as a sufficiently high concentration (3–5 mM) can temporarily inhibit cell growth, likely by reducing the electrochemical potential across the cytoplasmic membrane. When *E. coli* is grown in Luria Bertani (LB) medium, it can elevate the external indole concentration to 0.5–0.6 mM. Despite this, potentially inhibitory concentrations (1–5 mM) have been introduced to cultures to investigate the impact of indole on the expression of multidrug exporters, bacterial virulence, plasmid stability, and cell division. The natural ability of bacteria to produce these concentrations is unknown, so some of the effects observed in previous studies may be due to the use of artificially high levels of indole[6].

Two distinct modes of indole production can be observed: persistent and pulsed. In the persistent signaling mode, indole lingers in the culture for an extended period at a relatively low concentration (<1 mM). Conversely, pulsed signaling involves a rapid surge in intracellular indole concentration to a high level (50-60 mM) for a brief period (10-20 minutes) upon transitioning into the stationary phase. This temporary spike in concentration is likely due to the accelerated synthesis of indole, surpassing its removal from the cell through diffusion or membrane transporters[7].

A significant contrast can be observed in these modes is that persistent signaling is a consequence of the presence of indole in the growth medium. This type of signaling impacts both the producer cell and non-indole-producer cells in a similar manner. In contrast, pulse signaling is unique to the producer cell and occurs solely during the period of rapid indole synthesis[8]. In this study we focused on the production and excretion levels of indole by *E. coli* in a persistent mode, and how various metabolic and environmental factors played a role in determining the ultimate indole output in three different *E. coli* strains.

MATERIALS AND METHODS

Bacterial strains

In this study, three different *E. coli* strains were recruited, including the commensal (non-pathogenic) strain (ATCC8739) provided by the Egyptian drug authority (EDA), the pathogenic strain (ATCC 700728) purchased from the Faculty of Science at Helwan University, and the quality control strain (ATCC 25922) purchased from the Faculty of Agriculture at Ain Shams University.

Optimization of indole production by *E. coli*

Several approaches have been utilized to optimize the culture and incubation conditions. The process began with streaking bacterial strains from −80°C glycerol stocks on nutrient agar (NA) plates. Subsequently, a fresh single colony was inoculated into LB medium in 250-mL flasks and various volumes of LB medium (Sigma Aldrich, USA) were examined, ranging from 50 to 200 ml. Two sets of the same volume were prepared, with one set incubated in a static condition and the other in a shaking condition at 120 rpm. Both sets were incubated overnight, with each flask covered in aluminum foil or newspaper to maintain a dark environment due to the photosensitive nature of indole[9]. In line with established approach mentioned above, a fresh single colony was inoculated into preculture medium in 250-mL flasks and incubated under static conditions at 37°C for 18-24 h. Various volumes of LB medium were examined, ranging from 50 to 200 ml, with the overnight cultures subsequently diluted by a factor of 1:100, and vortexed to get a uniform suspension. The uniform suspension of bacteria was incubated again overnight under dark conditions at 37°C in shaking condition at 120 rpm[10]. The assessment of cell growth was evaluated by spectrophotometrically measuring the optical density at 600 nm (OD_{600}). In cases where the OD_{600} value surpassed 0.7, the culture sample underwent dilution to ensure it fell within the linear range of 0.2 to 0.7. The specific growth rates were ascertained by

measuring OD_{600} and then calculated using the linear segment of the natural logarithm of OD_{600} against time[11]. Indole yield was measured in both approaches, with the second approach resulting in the highest indole yield.
The preliminary trials aimed at identifying the optimal tryptophan concentration to enhance indole production involved supplementing LB broth with varying levels of tryptophan, ranging from 0.5% to 5% of the medium volume. The indole concentration was closely monitored for each concentration. Additionally, the temperature range of 30°C to 40°C was tested, and the pH was measured at various time intervals during the incubation period by collecting samples in conjunction with the assessment of indole yield[5].

Qualitative and quantitative analysis of indole

The indole assay kit (Sigma Aldrich, USA) is based on a modified version of Ehrlich's and Kovac's reagents, which reacts with indole to produce a pinkish-red compound at 565 nm. The intensity of this colored compound is directly proportional to the indole in the sample. This kit is suitable for indole determination in biological samples (indole produced by indole positive bacteria) as followed through manufacturer's recommendations. The kit protocol starts with preparing 1 mL of 100 uM Premix by mixing 10 uL of the standard indole (10 mM) and 990 uL of the blank medium. Then dilution of standards in 1.5 mL centrifuge tubes took place as described in **Table 1**.

Table 1 Standard indole dilutions

Tube	100 uM Premix	Growth medium	Indole uM
1	200 ul	0 ul	100
2	100 ul	100 ul	50
3	50 ul	150 ul	25
4	0 ul	200 ul	0

Then 100 uL of standards was transferred into separate wells of a clear, flat-bottom 96-well plate, followed by addition of 100 uL reagent to the four standards and the sample wells. The plate was tapped to mix thoroughly and was kept in dark condition for 10 min. The absorbance was measured at 565 nm (A565). Then the blank value (standard tube #4) was subtracted from the standard values and a plot of the A565 against standard concentrations. The slope was determined, and the indole concentration in *E. coli* culture supernatant was calculated as follows: Indole = (A565) sample – (A565) blank / Slope (uM^{-1}). The indole was quantified in *E. coli* culture supernatant (CS).

Statistical analysis

The statistical analysis was conducted using GraphPad Prism 5 software. The data from three distinct groups were assessed for various variables using one-way ANOVA. Each variable was analyzed individually across the three groups, with means presented alongside standard errors. Notably, each test was conducted in triplicate on independent days. Results of the ANOVA revealed statistical significance determined at $p< 0.05$* and $p< 0.01$**.

RESULTS

Indole production and quantification

The experimental setup that yielded the highest results across all bacterial strains tested involved the preparation of a bacterial suspension with an optical density (OD) between 0.2 and 0.7, which was then diluted 1:100 in fresh LB medium. The LB medium was supplemented with 1% tryptophan. Temperature conditions began at 36°C and were later increased to 38°C midway through the 18–24 h incubation period, with constant shaking at 120 rpm. The pH levels were continuously monitored as an indicator of the production of acidic products, which is directly linked to the bacterial yield of indole.

Under the experimental conditions described above, the *E. coli strain* (ATCC 700728) produced an average of 200± 50 uM of indole, whereas the other two *E. coli* strains (ATCC 8753 & ATCC 25922) displayed a lower average yield of 100± 50 uM of indole shown in **Figure 1.**

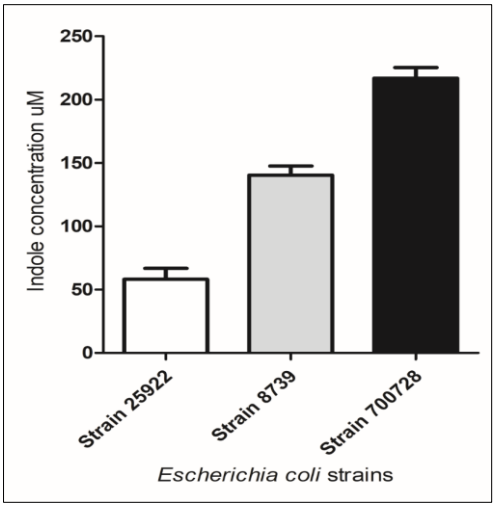

Figure. 1 The CS indole concentration in three *E. coli* strains. The comparison of the indole production capacity revealed that the pathogenic strain (ATCC 700728) displayed a notably higher concentration of indole under the experimental conditions employed.

Influence of temperature on indole production

Indole cell signaling is significantly influenced by temperature. Our study focused on assessing the effect of temperature fluctuations (30, 36, and 38 °C) on indole production. The results indicated a strong correlation between temperature and indole concentration. Notably, the highest temperature of 38°C led to a peak indole concentration of 200 ± 50 uM. Moreover, all three *E. coli* strains utilized in the experiment consistently maintained peak indole concentration at this elevated temperature. This suggests that *E. coli* may stimulate indole production at high temperatures as shown in **Figure 2**.

pH controls indole production

Throughout the incubation period, the pH was measured at regular intervals, showing variations in pH values from 8 to 5. These fluctuations indicated a transition from alkaline to acidic pH ranges. Additionally, it was observed that the concentration of indole increased with rising pH levels, reaching its maximum concentration under alkaline conditions. Conversely, a noticeable decrease in indole concentration was observed as the pH decreased towards acidity, likely due to the acidic nature of indole and its high concentration shown **Figure 3**.

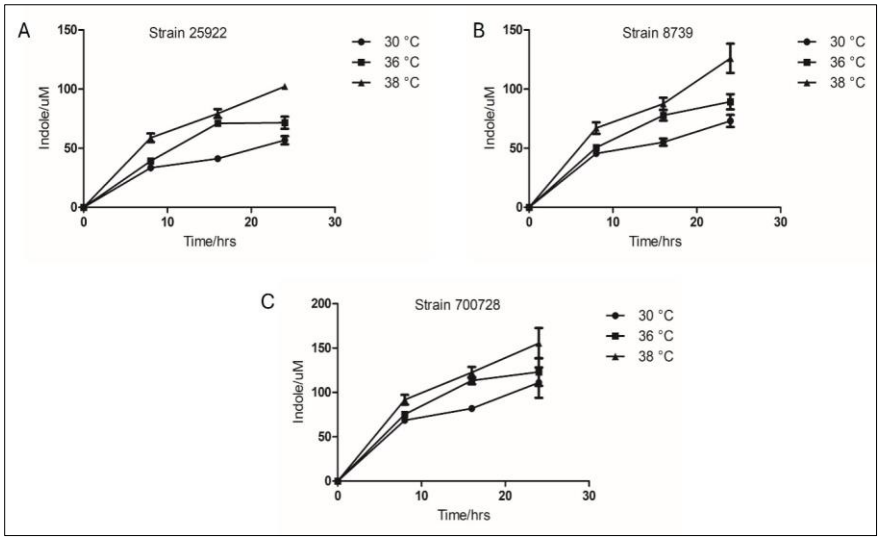

Figure. 2 Indole production after incubation at elevated temperature. The CS indole concentration of all three *E. coli* strains (A; strain 25922, B; strain 8739, C; strain 700728) consistently maintained its peak at elevated temperature 38 °C after overnight incubation $p< 0.05$*.

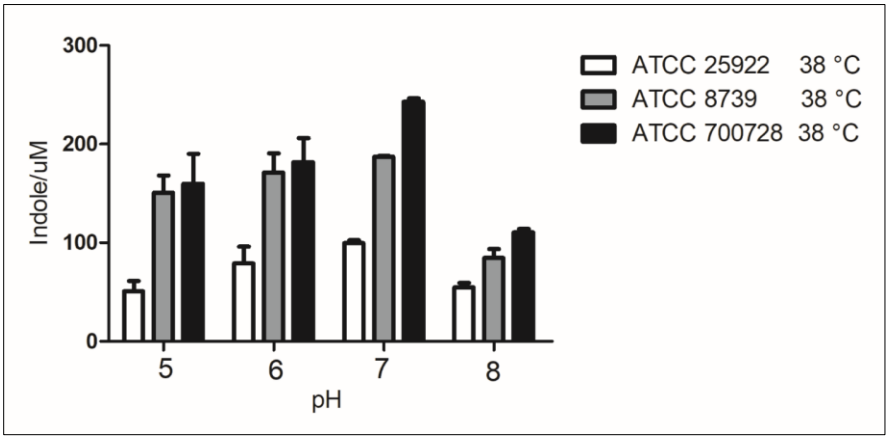

Figure 1. Effect of pH on indole production after overnight incubation in LB medium. Lower pH levels are associated with decreased indole production, whereas higher pH levels are linked to increased indole production $p<0.01$**.

DISCUSSION

The gut microbiota, a diverse microbial community found in the human intestinal tract, is composed of trillions of microbial cells and produces tens of thousands of detectable metabolites. However, many of these small molecules remain structurally and functionally uncharacterized [12]. *E. coli*, one of the most extensively studied gut bacteria, is estimated to be present in approximately 90% of the human population. *E. coli* isolates can act as commensals, probiotics, and pathogens, with certain strains like serotype O157:H7 contributing to serious and life-threatening infections worldwide. To facilitate their diverse interactions with other microbes and their animal host, *E. coli* strains produce a variety of small molecule metabolites, including indole, to mediate bacterial signaling[13].

TnaA is the enzyme in *E. coli* that catalyzes the reversible conversion of tryptophan into indole, pyruvate, and ammonia as part of the tryptophan pathway. *E. coli* employs repression, transcription attenuation, and feedback inhibition mechanisms to regulate the expression of the tryptophan (trpABCDE) and tna operons (tnaCAB) involved in tryptophan metabolism [14]. During the transition from the exponential phase to the stationary phase, there is a marked increase in the production of indole by *E. coli*. In the LB medium, the concentration of indole in the supernatant reaches its maximum at 0.5-0.6 mM in the stationary phase, thus earning the designation of a stationary-phase signal molecule[15].

In this study, the CS obtained from three different *E. coli* strains, the commensal (ATCC8739), the pathogenic (ATCC 700728), and the quality control strain (ATCC 25922) was subjected to evaluation regarding indole yield. The comparison of the indole production capacity revealed that the pathogenic strain displayed a notably higher concentration of indole under the experimental conditions employed producing an average of 200± 50 uM of indole.

The presence of extracellular indole may be significantly influenced by various environmental factors, including cell population, carbon sources, temperature, and pH. In the initial optimization process, the quantification of indole was carried out while studying and optimizing each variable. The quantification was performed using a modified Kovac assay with an indole assay kit. It was observed that the concentration of extracellular indole is dependent on cell population density, with *E. coli* producing indole during the early exponential phase[16]. The concentration peaks at around 200± 50 uM for pathogenic strain (ATCC 700728) while for the commensal (ATCC 8739) and the quality control (ATCC 25922) strains, the peak was at 100± 50 uM in the stationary phase and remains relatively stable throughout this phase.

LB broth stands out as the medium of choice due to its well-balanced composition of nutrients that promote the growth and metabolism of *E. coli*. The presence of peptone and yeast extract in appropriate concentrations caters to the nutritional needs of *E. coli*. The slightly alkaline pH of the medium falls within the ideal range for *E. coli* growth, facilitating optimal enzymatic functions and metabolic activities. Additionally, its strong buffering capacity enables it to withstand pH fluctuations caused by bacterial metabolism or external factors. These characteristics collectively establish LB broth as a dependable option for the routine cultivation of *E. coli*[17].

It was evident during our initial trials that the yield of indole was suboptimal without supplementing LB medium with tryptophan with an average of 75± 25 uM regarding the commensal (ATCC 87390) and the quality control (ATCC 25922) strains, while an average of 130± 20uM was observed with pathogenic strain (ATCC 700728). While LB broth is rich in nutrients, including tryptophan, our preliminary trials revealed that composition of the medium needed optimization, specifically requiring supplementation with 1% tryptophan. This decision was crucial to ensure consistent and sustained levels of tryptophan throughout the experiment which is essential for indole production mediated by the enzyme TnaA in *E. coli*.

The study suggests that optimal indole production in *E. coli* requires adequate exogenous tryptophan availability. Higher tryptophan levels increase indole production, as the amount produced correlates with the concentration supplied to the culture. Supplementing the medium with 1% tryptophan ensures consistent substrate availability for TnaA-mediated indole production.[5]

Here, we reported that higher temperature and pH levels were linked to increased levels of indole production. The significance of temperature in indole signaling and production is evident from several studies. For example, the induction of *tnaAB* in *E. coli* occurred when the temperature was shifted from 30°C to 43°C [18]. Additionally, mutants lacking heat shock proteins IbpA and IbpB showed increased extracellular concentrations of indole. Notably, *E. coli* was able to produce indole even at 50°C, which contrasts with previous findings indicating a loss of indole production at 44.5°C after 6 days. This difference may be attributed to variations in culture times (16 h at 50°C vs. 6 days at 44.5°C) and strains (BW25113 vs. ATCC 11775)[19].

Through the initial characterization process aimed at optimizing the variables under investigation, a consistent decrease in pH was noted during the incubation period. This decrease was linked to the production of indole, with the observed correlation being associated with the rapid increase in indole yield. This observation suggested that this abrupt rise in indole

production may have interrupted the production cascade, potentially due to the acidic nature of indole. In addition, it was revealed that low pH levels inhibit indole production, while high pH levels enhance indole production, both at temperatures of 30°C, 36°C, and 38°C.

The findings are consistent with previous investigations aimed at understanding this phenomenon where low pH inhibits indole production in *E. coli*, and TnaA was one of the most induced proteins at pH 9.0 [20]. Transcriptomic data showed that *tnaA* was repressed under acidic conditions, while it was induced under basic conditions[21]. It was even proposed that TnaA could counteract alkaline stress, such as trimethylamine N-oxide, as evidenced by the low survival of an indole-mutant (ΔtnaLAB) in trimethylamine N-oxide [11]. Our study clearly demonstrated that *E. coli* produces more indole at high pH. These results provide further support for the hypothesis that *E. coli* may deactivate *tnaA* in the acidic stomach and activate it in the basic gut [13].

CONCLUSION

This investigation unveiled the metabolic and environmental cues that influence and regulate the production of indole by *E. coli*, establishing a direct link between tryptophan concentration in the culture medium, pH levels, and temperature with enhanced indole production. However, the precise determinants of indole production remain poorly understood. Future approaches should focus on elucidating the intricate pathways and control mechanisms governing indole biosynthesis by broadening pH and temperature ranges beyond those currently specified in our study, employing acidic and basic buffers to mitigate pH variations. Moreover, it is crucial to conduct an in-depth analysis of the incubation conditions, determining whether the use of conventional shaker incubators or fermenters would yield more accurate and reliable monitoring of the conditions implemented for indole production.

Funding Acknowledgment

No external funding was received.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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