L-tryptophan production by *Escherichia coli* in the presence of Iranian cane molasses

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ABSTRACT

The essential amino acid L-tryptophan (Trp) has importance as a pharmaceutical agent, especially in neuro-medicine. It is also added to feed products as a food fortifier. Furthermore, application of Trp is widespread in biotechnology. Trp is produced by a condensation reaction between indole and L-serine, which is catalyzed by bacterial tryptophanase activity. In this study, we have investigated Trp production using microbial system in the presence and absence of its precursors and Iranian cane molasses. The results showed that the optimum concentration of the molasses for maximum bacterial growth is 10 g/lit. Furthermore, in order to assay the amount of tryptophan produced, thin layer chromatography was used. The results showed that Iranian cane molasses contains considerable amounts of serine and indole, enough for Trp production (0.48 mM) in culture medium. But additional indole has inhibitory effects on Trp production. The data are compatible with previous reports on inhibitory effect of indole not only on cell growth but also on tryptophanase formation and function.

Keywords: Tryptophan; Tryptophanase; Indole; Cane Molasses; Escherichia Coli.

INTRODUCTION

L-tryptophan (Trp) is an essential aromatic group amino acid with the unique indole side chain (Figure 1) that makes it a fundamental precursor to a number of neurotransmitters in brain, i.e. serotonin [1], melatonin [2] and niacin [3], which are essential for the regulation of appetite [4], sleep [5], mood [6] and pain level [7].

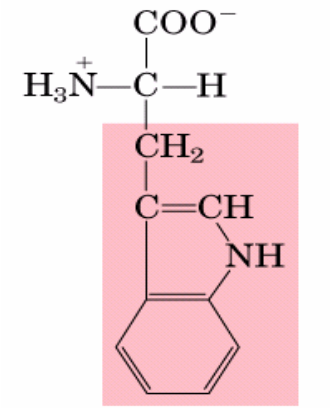

Figure 1. The structure of L-tryptophan

In addition, Trp is an important and frequently used starting material in the chemical synthesis of a range of pharmaceuticals which are known as antidepressants [8] and in the treatment of schizophrenia [9]. Industrial production of L-tryptophan is mostly for feed and pharmaceutical purposes. The first reports on L-tryptophan production date back to the beginning of the 20th century [10]. To meet the increasing demand, a wide variety of chemical and biotechnological methods have since been developed. Chemical synthesis was the first methods applied to an industrial scale process [11]. By the end of the 80s, production of L-tryptophan was carried out through chemical, enzymatic and fermentation processes [12].

The enzymatic production of L-tryptophan from precursors involves a single reaction step. It may be performed with isolated enzymes, either tryptophan synthase (TSase; EC 4.2.1.20) or tryptophanase (TPase; EC 4.1.99.1), or by a variety of microorganisms with these enzyme activities [13]. *E. coli* has both the enzymes tryptophanase and tryptophan synthase [14]. TSase exists in two distinct forms including trptophan-indusible synthase (TSase-Ti) and the tryptophan-repressible tryptophan synthetase (TSase-Tr) [15]. TSase-Tr uses the precursor indole-3-glycerol phosphate which was existed from an intermediate substrate such as chorismate. This intermediate component was produced from shikimate. TSase-Ti catalyzes the synthesis of tryptophan according to reaction1:

L-serine + indole ⟶ L-tryptophan + water (1)

TPase is a multifunctional enzyme which catalyzes the conversion of L-tryptophan to pyruvate, ammunia and indole (reaction2):

L-tryptophan + H_2O ⟶ Pyruvate +NH_3 + Indole (2)

As well as synthesis of L-tryptophan from indole, pyruvate and ammonium chloride (reaction 3):

Indole + Pyruvate + NH_3 ⟶ L-trytophan + H_2O (3)

Attempts have been made to overproduce this important amino acid in a wide number of bacteria including Escherichia coli [16], Bacillus subtilis [17], Brevibacterium flavum [18] and Pseudomonas aeruginosa [19]. Biotransformation uses *E. coli* cells, which overproduces tryptophan synthase, indole and L-serine as substrates. The majority of tryptophan production, however, is by microbial fermentations mainly with Corynebacterium and *E. coli* [15]. Microbial fermentation strains allow the production of various amino acids from cheap and renewable carbon sources such as sucrose, glucose or molasses; therefore, they are usually more favorable than biotransformation processes [20].

In this study, it is argued that cane molasses can be good material not only as a carbon source but also serine source for Trp production by *E. coli*. Dual role of indole in Trp production has also been investigated.

MATERIALS AND METHODS

Chemicals

L-tryptophan, L-serine and indole were purchased from MERCK and cane molasses, a by-product from sugar production, was prepared from sugar factory.

Microorganism

The microorganism used was *E. coli* (ATCC 11303), from Iranian Research Organization for Science and Technology (IROST).

Standard Preparation

Standard stock Solutions of tryptophan (0.001M) was prepared in 10% (V/V) n-butanol and stored at 5°C. The stock solutions were prepared every 3 weeks [21]. The stock solutions were immediately prepared for experiment. Standard stock solution of serine (0.001M) was prepared in water.

Ninhydrin Solution

Ninhydrin solution (0.1% W/V) was prepared in 100 ml acetone [22].

Protein Analysis

Protein production was determined by the Bradford method, with bovine serum albumin as a standard. One milliliter of culture medium was used to protein assay per hour [23]. This assay is very rapid so that dye binding process was completed in approximately 2 min with good color stability for 1 hour.

Sugar Analysis

The total Sugar was determined by the Phenol sulfuric acid method, with sucrose sugar as a standard. One milliliter of culture medium was used to sugar assay per hour [24]. In the sugar assay, because of the high sample concentration, taken samples were diluted 40 folds.

Instrumentation

Spectrophotometer (CECIL 9000, England) was used for protein and sugar assay. Refractometer (SCHMIDT, HAENSCH model) was applied for measuring total soluble solids, and polarimeter (SCHMIDT, HAENSCH model) was used for the determination of the amount of sucrose in the cane molasses. Scane TLC system was CAMAG TLC scanner II. CATS 3 Software was used for analyzing the data.

Culture Medium

Lyophilized cells of *E. coli* strain were grown on nutrient agar 0.5% (W/V) NaCl slant at 4oC. Inoculums was prepared by inoculating one loopful of culture into 100ml broth medium containing 0.5% (W/V) each of yeast extract and NaCl, 1% (W/V) each of peptone and glucose, 0.5% (W/V) ammonium sulfate (pre-culture 1) and incubated at 37oC for 6h on a rotary shaker at 150 rmp. Then, the cells of pre-culture 1 were transferred to the same medium and were also incubated in the same conditions (preculture 2).

The actual determination of tryptophan was carried out by inoculating 1 ml of the cells of pre-culture 2 into 100 ml Erlenmeyer flasks containing 1% (W/V) peptone, 0.5% (W/V) yeast extract, 0.5% (W/V) NaCl, 0.05% (W/V) ammonium sulfate and 10 g/l cane molasses (sample 1), as well as the same culture medium instead of cane molasses, 1% (W/V) sucrose (sample 2), 0.1% (W/V) indole and 1% (W/V) sucrose (sample 3), 0.1% (W/V) indole and 10 g/l cane molasses (sample 4), 0.1% (W/V) indole, 10 g/l cane molasses and 0.2% (W/V) serine (sample 5), 0.1% (W/V) indole, 1% (W/V) sucrose and 0.2% (W/V) serine (sample 6) were used in the experiment.

Besides, in order to prohibit from Maillard reaction, cane molasses were sterilized

separately at 121oC for 15 min, or they were passed through a 0.22 μ Millipore filter. After centrifuging cells (20 min in 14000g, 4°C) [25], the supernatants were prepared for TLC and scan TLC.

In order to investigate the need to the carbon source for tryptophan production, 1 ml of pre culture 2 was inoculated into the above medium containing just 10 g/l cane molasses and any additives. The cane molasses concentrations were measured as W/V (not V/V) because of their high viscosity, and thus high error in volume measurement and transferring. Then, the cell suspension was centrifuged and subsequently, the biomass was washed with a 0.9% (W/V) NaCl solution and re-centrifuged [15]. Next, the supernatant was existed. All tryptophan determinations were done on supernatants of rest cell.

Growth of Cells Measurements

Growth in the test medium was evaluated by periodically measuring OD_{620} in a spectrophotometer per hour.

Dry Cell Concentration Determination

Samples that were used in per hour for growth curve were centrifuged (14000g) for 20 min. The pellet was suspended in distilled water and re-centrifuged. Biomass was determined by weighing after drying at 65°C for 24 h.

TLC Method

The amino acid produced was identified by thin-layer chromatography on silica gel 60 F_{254} plates (MERCK) with a solvent system of n-butanol/acetic acid/water (65:13:22 V/V/V) [27]. Briefly, the supernatant from centrifugation of culture medium was mixed with n-butanol solvent (1:2). The mixture was vigorously vortexed and then incubated at room temperature for 3hrs so that two phases were formed. After separating organic phase and its evaporation, 50 μl of the organic phase was loaded for TLC with the help of Hamilton syringe [28]. The chromatogram was developed in rectangular glass chamber pre-equilibrated with the solution system for 15 min. Then the chromatogram was dried using hair dryer and a solution of the ninhydrin reagent was sprayed on the TLC. Then, chromatogram was dried for 5 min at 110°C. The identification was confirmed by R_f comparison of tryptophan standard and sample (Figure 4a).

TLC scanning method

To measure tryptophan concentration in each sample, TLC scanning method was performed in the wavelength of 254 nm (LINOMAT IV model) [29]. The scanning results were analyzed using CATS 3 software to culture peak areas (Figure 4b).

Statistical analysis

All experiments were carried out three times, and the mean, standard error and P-values were calculated using SPSS (version 16.0) Software.

RESULTS

Determination of tryptophan concentration in medium

Because yeast extract and peptone in culture media might contain tryptophan and interfere on results, the existence of tryptophan was checked in yeast extract and peptone solution (0.5% and 1%, respectively) by TLC and its scan. The concentration of tryptophan was measured as 0.22 ± 0.05 mM and 0.14 ± 0.03 mM for yeast extract and peptone, respectively.

Determination of cane molasses composition

The percentage of dry mass (°Bx), the percentage of sugar (Pol), the percentage of sugar purity (Q) and pH were measured for Iranian cane molasses (Table 1).

Table 1. Iranian cane molasses composition

°Bx (%)[a]	73.4
Pol (%)[b]	38.6
Q (%)[c]	52
pH	5.1

a) Total soluble solids (w/w)
b) Sucrose content (g/100ml cane molasses)
c) Purity percentage = (Pol / °Bx) × 100

Determination of optimum cane molasses concentration for cell growth

In order to determine the optimum cane molasses concentration for cell growth, the amount of bacterium biomass was measured in different concentrations of the cane molasses (Figure 2). Cane molasses concentrations were expressed by percentage of biomass weight per volume (%W/V) because of its high viscosity. In each molasses concentration of media, cell suspension of stationary phase was

centrifuged and weighted. The molasses concentrations corresponding to the highest biomass amount was selected as optimum cane molasses concentration for bacterial growth. The results are mean values of triplicates. As it can be seen in figure 2, the maximum amount of biomass was obtained using 10 g/l cane molasses concentration. Therefore, the next experiments were performed using 10 g/l cane molasses concentration.

Investigation of *E. coli* metabolism

To survey the level of metabolism, protein and sugar concentrations were determined in the culture medium during bacterial growth (Figure 3). The cells were grown in the presence indole (0.1% W/V) and cane molasses (10 g/l) as carbon source at 37°C. In the case of protein, Bradford Method was used. For this purpose, a small aliquot of culture medium was hourly assayed for protein using Bradford method. The binding of dye to protein causes in absorption maximum of dye in 595 nm. Moreover, a small aliquot of culture medium was assayed for sugar using phenol sulfuric acid method by following A_{490}, hourly. The results are mean values of 0observed that sugar and protein concentrations decreased and increased, respectively per hour until maximum cell growth (as changes in A_{620} and weight of biomasses show). The cells in the stationary phase were used for next experiments.

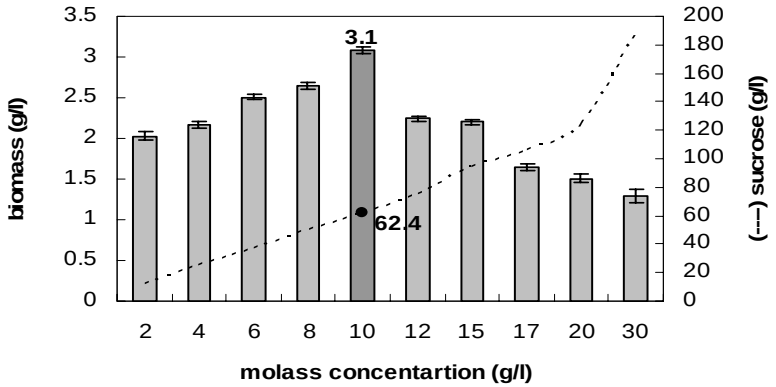

Figure 2. Determination of optimum cane molasses concentration for *E. coli*.

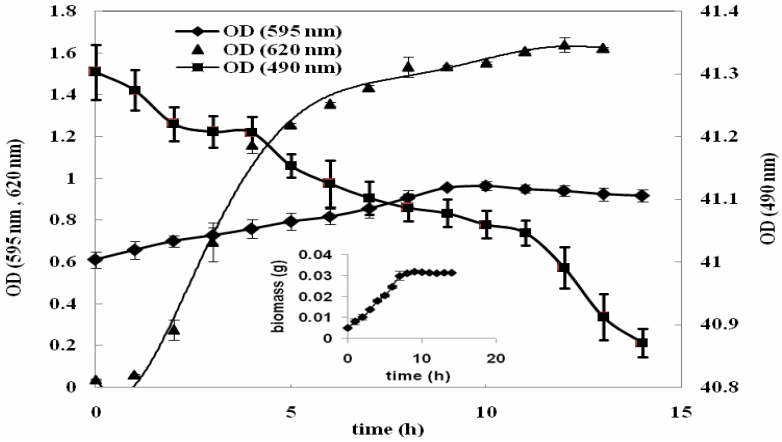

Figure 3. Growth, protein and sugar curve of *E. coli.* ◆ (Protein curve), ▲ (growth curve), ■ (sugar curve). Inset: Growth curve using weight of biomass.

Effect of precursors on tryptophan production monitored by TLC

Tryptophan production was followed using TLC in the presence and absence of its two precursors (indole and serine) in medium containing either sucrose or molasses on cell growth. These precursors were added to culture medium after 6 hours of cell culture with the cultures in stationary phase. After staining by ninhydrin, the tryptophan spots (purple-brown) in TLC pattern are circular, of diameter 2.5-3.0 mm (Retention factor, R_f=0.54) and are distinguishable from the spot Corresponding to phenylalanine (purple)

which is much closed to tryptophan spot (Figure 4a). In order to investigation of spot of tryptophan, indole and PLP standard (as negative control) were also used. The results showed that ninhydrin reagent is linked only to the primary type amine of tryptophan and the secondary amine in indole as well as the ternary amine of PLP is not detected using this method (data not shown). Phe, Trp and Ser are standard samples. Scanning of TLC chromatograms and calculating the peak areas provided the amount of tryptophan produced in the samples using standard curve. Figure 4b shows a typical TLC scan and tryptophan peak area as an example. Tryptophan production by *E. coli* in the presence and absence of its precursors (0.1% indole, I; 0.2% serine, Ser) as well as 1% sucrose (Suc) or 10 g/lit molasses (M) are shown in Figure 5. The culture medium components and their concentrations have been mentioned in Materials & Methods as sample 1-6. The significance of differences between each of the two given conditions has been expressed as P-value calculated using SPSS Software.

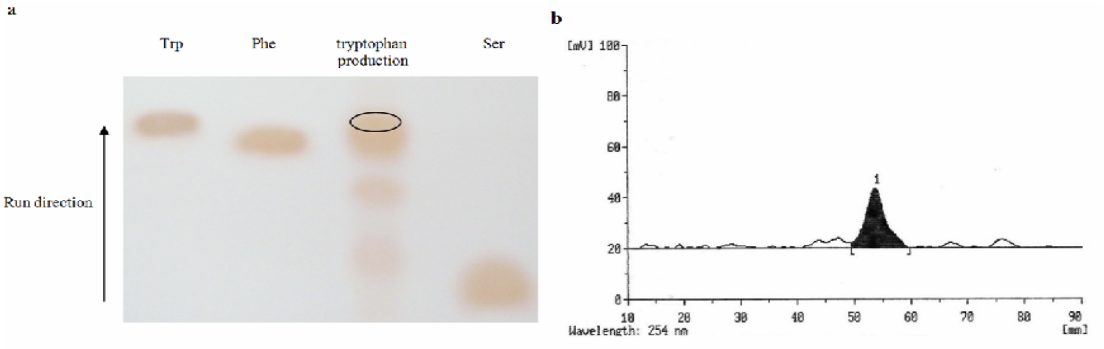

Figure 4. a) TLC pattern of amino acid production in culture medium. **b)** A typical TLC scan and tryptophan peak area as an example.

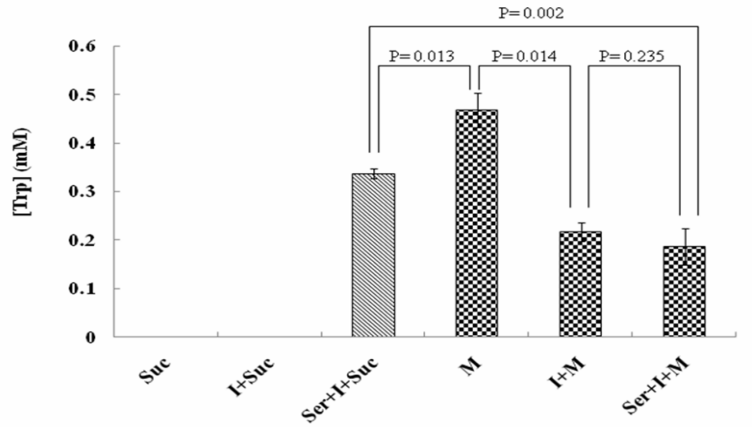

Figure 5. Trp production by *E. coli* in the presence and absence of its precursors

DISCUSSION

Tryptophan is an amino acid necessary for growth in infants and for nitrogen balance in adults. It is an essential amino acid, which means body cannot produce it; hence, we must get it from our diet.

As cane molasses are cheaper than other carbon source, and are mainly composed of sucrose and glucose it is good candidate for bacterial growth. The optimum cane molasses concentration for *E. coli* growth is 10 g/l which is corresponding to 62.4 g/l sugar concentration in culture medium (Figure 2). According to the data, biomass started to decrease in molasses concentrations more than 10 g/l. It is probably related to growth inhibitory effect of sugar component of the molasses, because of decrease in hydrolysis rate of sugar or osmotic effect of sugar high concentration [30]. The optimum molasses concentration (10 g/l) was used in the next experiments.

Increase in protein concentration and decrease in sugar content of medium, during the bacterial growth demonstrate active metabolism of *E. coli* (Figure 3). That means the bacteria are consuming sugar of the molasses (the only carbon source in medium) and producing protein. Thus, it can be concluded that the bacteria are in the best situation for producing metabolites (like

tryptophan) during the stationary phase. The bacteria in next experiments were in stationary phase. On the other hand, the data show that *E. coli* can grow and synthesize its proteins without adding precursors of tryptophan synthesis (indole and serine) to medium. This might be due to the presence of enough tryptophan for growth in yeast extract (0.22 ± 0.05 mM) and peptone (0.14 ± 0.03 mM) as components of culture medium, because *E. coli* has been reported to be tryptophan auxotroph [25].

Existence of cane molasses in cell culture caused interesting results (Figure 5):

1. Although sucrose is a suitable carbon and energy source for cell growth [31], it is not precursors (indole and serine) are required for this purpose as well.
2. It seems that Iranian cane molasses contains adequate amounts of both indole and serine, so that no need to add the precursors in presence of molasses. The amount of indole and serine in molasses are enough to produce 0.47 mM tryptophan in culture medium.
3. Although indole is the precursor of tryptophan production by tryptophanase, it also has inhibitory effects either on bacterial growth or tryptophanase production [32]. This can explain why indole has caused a statistically significant decrease (53.7%) in tryptophan production (P=0.014). The same effect can be observed in the case of all media containing indole.
4. Serine has not shown a significant effect on tryptophan production (P= 0.235).
5. Comparison of medium containing sucrose with medium containing molasses both in the presence of precursors shows that molasses causes a significant decrease in tryptophan production (44.8%, P=0.002). This might be due to a lot of substances in molasses which have probable inhibitory effect on tryptophan production.

In summary, our results show that the optimum concentration of the molasses for maximum bacterial growth is 10 g/lit. Moreover, Iranian cane molasses contains considerable amounts of serine and indole, enough for Trp production (0.48 mM) in culture medium. This is a very important result showing less need to add serine and indole. But additional indole has inhibitory effect on Trp production. The data are compatible with previous reports on the inhibitory effect of indole not only on cell growth but also on tryptophanase formation and function.

ACKNOWLEDGEMENTS

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