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Tryptophan Oxidative Metabolism Catalyzed by *Geobacillus Stearothermophilus*: A Thermophile Isolated from Kuwait Soil Contaminated with Petroleum Hydrocarbons

Jassim M. Al-Hassan, Samira Al-Awadi, Sosamma Oommen, Abdulaziz Alkhamis and Mohammad Afzal

Department of Biological Sciences, Faculty of Science, Kuwait University, Safat, 13060, Kuwait.
Corresponding author email: afzalq8@gmail.com

Abstract: Tryptophan metabolism has been extensively studied in humans as well as in soil. Its metabolism takes place mainly through kynurenine pathway yielding hydroxylated, deaminated and many other products of physiological significance. However, tryptophan metabolism has not been studied in an isolated thermophilic bacterium. *Geobacillus stearothermophilus* is a local thermophile isolated from Kuwait desert soil contaminated with petroleum hydrocarbons. The bacterium grows well at 65 °C in 0.05 M phosphate buffer (pH 7), when supplied with organic compounds as a carbon source and has a good potential for transformation of steroids and related molecules. In the present study, we used tryptophan ethyl ester as a carbon source for the bacterium to study the catabolism of the amino acid at pH 5 and pH 7. In this endeavor, we have resolved twenty one transformation products of tryptophan by GC/LC and have identified them through their mass spectral fragmentation.

Keywords: tryptophan, kynurenine pathway, metabolites, *Geobacillus stearothermophilus*, GC/MS

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Introduction

Tryptophan (Trp) is an important essential aromatic amino acid with a daily recommended requirement of 4 mg/kg body weight in humans with clinical implications.^{1,2} L-Trp is a precursor for many compounds such as niacin, and actinomycins³ and its metabolism leads to many metabolites that may be physiologically valuable or even toxic.⁴ These molecules may include quinolinic acid, indoleacetic acid, indole, indoxyl, skatole and numerous other compounds.⁵⁻⁷ Metabolic products of tryptophan such as serotonin upregulates appetite, sleep pattern and mood.⁸ Its degradation via the kynurenine is the major degradative pathway leading to *de novo* synthesis of nicotinamide adenine dinucleotide (NAD⁺),⁹ which is a critical coenzyme for cell viability in liver and other organs including kidney, central nervous system and pineal gland.¹⁰⁻¹² Kynurenine pathway (KP) is also the major regulatory pathway for immune response and many other diseases such as dementia, Parkinsonism, schizophrenia, Huntington's disease, brain tumor and multiple sclerosis.¹³ Over the past several years, KP has been studied by many researchers for its role in neurological diseases.¹⁴ KP enzyme mediated Trp metabolism involves dioxygenases such as indoleamino 2,3-dioxygenase (IDO)-1, tryptophan dioxygenase (TDO) and IDO-2.^{15,16} TDO is a recognized catalyst of the former and rate-limiting step in the metabolism of tryptophan (Trp) along the kynurenine (Kyn) pathway. Trp as such has been therapeutically used in the treatment of a variety of conditions mostly insomnia, depression and anxiety etc.^{17,18}

Soil bacterial metabolism of Trp has also been studied for its conversion to auxins such as indole 3-acetic acid (IAA) for plant growth and development.^{19,20} Although many Trp metabolites have been identified in soil,²¹ IAA is the only metabolite that retains its indole structure.²² In soil bacteria, it is not only the L-Trp that is catabolized into IAA, but many researchers have suggested that D-Trp may also be converted into auxin IAA by microbiota²²⁻²⁵ where L-Trp aminotransferase (L-TAT) plays a major role in the transformation of Trp to IAA.²⁶ However, for the transformation of D-Trp, it requires TAT with a special D-stereospecificity to produce IAA. It is known that D-amino acids are a part of the bacterial cell wall and antibiotics elaborated by actinomycetes.²⁷ Martens and Frankenberger²⁸ have indicated that 80% of the

L-Trp carbon added to the soil is evolved as CO₂ after 5 days of exposure suggesting the majority of L-Trp added to the soil is utilized by the kynurenine pathway (KP). This may be through degradation of Trp catalyzed by indoleamine 2,3-dioxygenase resulting in the formation of intermediates such as kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, and NAD⁺.^{15,16}

It has been reported that the ability of different soils to catabolize Trp into IAA may be different.²⁹ Kuwait desert soil bacteria have not been studied before for catabolism of Trp into IAA, its hydroxylated products and other metabolites. A study of this transformation is of particular interest because of the harsh climatic nature of Kuwait desert where most bacteria are thermophiles rather than mesophiles. Our objective was to study a thermophile, isolated from Kuwait soil contaminated with petroleum hydrocarbons, for its potential to catabolize Trp into IAA and other metabolites. The second objective of this study, besides the identification of metabolites, was to unravel the Trp metabolic profile using a purified thermophile avoiding any synergistic effect of other soil microbes.³⁰

Materials and Methods

L-tryptophan, L-tryptophan ethyl ester and various KP metabolites were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). All solvents and buffer chemicals were of HPLC grade purchased from Fluka and Riedel-de Haën. (Switzerland). Inorganic salts were purchased from Fluka and Riedel-de Haën (Germany) and Merck (Darmstadt, Germany). Tryptone, yeast extract and agar were from Difco Becton Dickinson and company (Sparks USA) and Scharlau (Barcelona, Spain). Kieselgel-60 F₂₅₄ fluorescent thin layer chromatographic plates (TLC) were obtained from Merck (Darmstadt, Germany).

Organism

The thermophilic *Bacillus* identified as *Geobacillus stearothermophilus* was isolated from crude oil contaminated soil of Burghan oil field in Kuwait. The bacterium was purified many folds and was maintained on tryptone yeast extract (TYE) media. Its optimum growth parameters were studied using a range of temperatures from 50–65 °C, and different pH (4–8) in 0.05 M Phosphate buffer. Its identification was confirmed by the National Collection of Industrial and

Marine bacteria, Terry Research Station, Aberdeen, Scotland, UK. A reference specimen was submitted in the culture collection facility of the Faculty of Science, Kuwait University.

Tryptophan transformation studies

The strain was maintained at 4 °C on tryptone and yeast extract agar plates as described previously.^{31–36} Starter cultures, (50 mL) of *G. stearothermophilus* were grown on TYE medium for 14 hrs in a shaking incubator at 65 °C. The cultures were transferred to fresh 500 mL TYE media in 2 L baffled flasks and were shaken at 65 °C until mid-log phase of growth (3.5–5 hrs). The cells were collected by centrifugation at 8000 rpm for 20 min. The cells were washed and suspended in 5–10 mL of 0.05 M phosphate buffer (pH 7). The suspension was transferred to 200 mL of the buffer solutions at pH 7 and pH 5 containing 10% of Castenholz mineral salt solution.³⁷ Because of tryptophan solubility problem, an aqueous solution of L-tryptophan ethyl ester was added to achieve a final concentration of 1 mg/mL of L-tryptophan ethyl ester and the flasks were re-incubated at 65 °C for a period of 72 hours. Standard L-tryptophan ethyl ester and tryptophan were also examined under the same experimental conditions, to check their aerial oxidation in the test media. However, no metabolites were observed in these experiments confirming that all tryptophan products were bacterial catalyzed metabolites.

Extraction and isolation of transformation products

The incubated cultures were freeze dried after adjusting the pH to 6.8. The freeze dried material was extracted with acetonitrile by stirring it overnight at 40 °C and then with acetonitrile:water (70:30 V/V). The pooled extract, after filtration, was evaporated under reduced pressure on a rotary evaporator. The concentrated extract was re-dissolved in acetonitrile:water (70:30 V/V) and chromatographed on TLC plates using benzene:ethyl acetate:acetone:methanol:water (22:20:3:3:2 V/V) and also butanol:acetic acid:water (2:1:1 V/V) as mobile phase. Two well resolved bands, as visualised under UV light (λ 254 nm), were marked and a two inch side strip of the 20 × 20 cm chromatograms was stained with ninhydrin solution made in aqueous acetone. The TLC strip, after heating, developed colored bands corresponding to amino

group bearing components. Corresponding bands from the TLC plates were scratched, pooled and re-extracted into acetonitrile:water (70:30 V/V). The concentrated extracts were re-chromatographed on TLC till both compounds were obtained in pure state. Most other metabolites could not be resolved on TLC plates in the above mentioned mobile phases. Although many metabolites, present in the crude mixture could not be separated by liquid chromatography by the procedure described by Martens and Frankenberger,³⁸ all metabolites could be resolved by GC as shown in Figure 1.

Analytical methods

¹H and ¹³CNMR spectral data were used when sufficient material was available for spectroscopy. The data were collected using a 600 MHz NMR (Bruker AC 600) instrument as methanol-d₄ solution. Two GC/MS systems were used for analyses of L-Trp metabolites and instrumentation and experimental conditions are given in Table 1.

A pre-column derivatization of amino acids was obtained with phenyl isothiocyanate (PITC) according to the recommended procedure of Waters Pico-Tag method. Derivatization of amino acids was also carried out with [N,O-bis(trimethylsilyl) acetamide] BSA; [N,O-bis(trimethylsilyl) trifluoroacetamide] BSTFA; and trimethylchlorosilane, BSTFA + TMCS.

ESI-LC/MS spectra were obtained using Thermo LCQ Fleet LC/MS equipped with a Thermo Hypersil Gold column 10 cm × 2.1 mm, particle size of 5 μ . Linear gradient elution was carried out using acetonitrile changing over to acetonitrile:water (90:10 V/V) in 45 minutes at a flow rate of 0.8 mL/minute.

Results and Discussion

For optimizing the incubation time, a time course study was initiated by incubating Trp with *G. stearothermophilus* for 24, 48 and 72 hours and it was found that better yields of the metabolites could be obtained after 72 hours of incubation. Purified metabolites and their PITC, BSTFA + TMSC derivatives were identified by GC complimented with mass spectrometry and LC/MS. The major tryptophan metabolites, identified through MS fragmentation were as follows: 5-hydroxytryptophan, indole-3-carboxylic acid, 5-hydroxyindole acetic acid, indole acetic acid (IAA), nicotinic acid, o-aminohippuric acid, 3-indole carboxylate, 3-indole acetamide, 3-indole lactic acid,

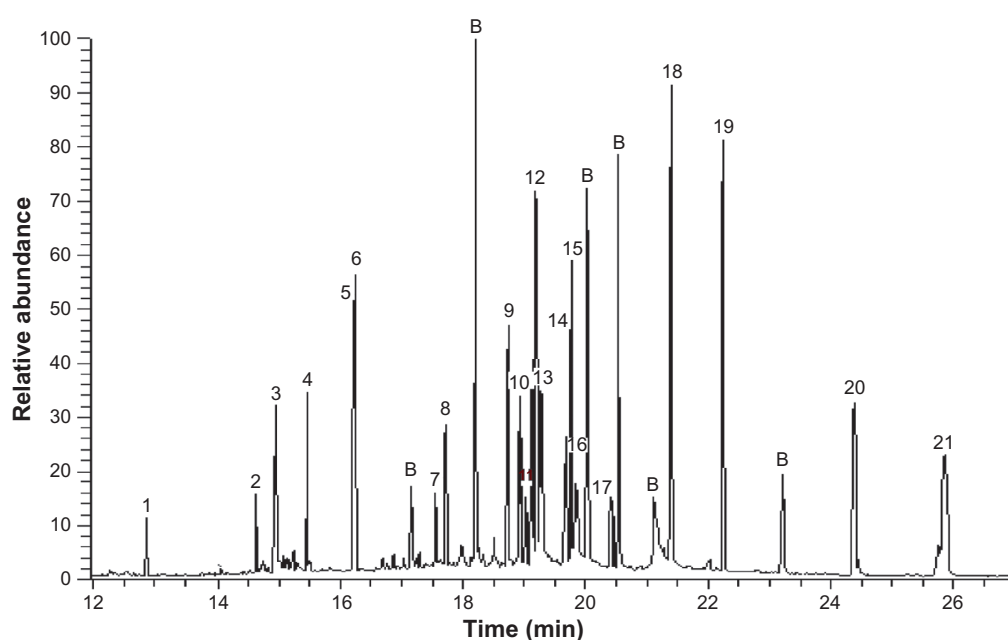


Figure 1. Gas Chromatogram of tryptophan metabolites.

3-indolepyruvic acid, kynurenine, 3-hydroxykynurenine, N-formylkynurenine, N-acetylkynurenine, quinolinic acid, serotonin, melatonin (serotonin and melatonin were present in trace amount in biotransformation experiments carried out at pH 5), N-acetylserotonin, and 2-amino-3-oxoprop-1-enyl-fumaric acid (Fig. 1). Incubation at buffer pH 7 yielded IAA, serotonin, N-acetylserotonin and melatonin, at slightly higher yield, as biotransformation

products. All these metabolites were confirmed through their MS fragmentation pattern.

Elemental composition was calculated for the molecular or pseudo molecular ion as well as for fragment ions and neutral losses observed in the ESI-MS spectra. Further information, about the number of double bonds and rings, was obtained from the elemental composition of the compounds.

Table 1. Details of the instrument and experimental conditions used in the analyses.

Equipment	System-1	System-2
GC	Trace GC ultra	Agilent 6890
Inlet	Split/splitless	Split/splitless
Detector	GC/MS DFS (Bremen, Germany)	Agilent MSD-5973
Autosampler	TR1 plus Thermo	Agilent 7683
Liner	Split (P/N: 453T1905)	Split (P/N 5183-4647)
Column	TR5MS-30 m × 0.25 mm × 0.1 μm	OV1-30 m × 0.25 mm × 0.1 μm
Experimental conditions		
Inlet temp.	280 °C	250 °C
Transfer line	290 °C	270 °C
Injection vol.	1 μl	0.2 μl
Split ratio	Splitless	1/50
Carrier gas	Helium	Helium
Gas flow	0.8 ml/min Initial 60 °C-3 min hold; 10 °C/min ramp to 250 °C hold 15 min; 10 °C ramp to 280 °C; 10 min hold	1.0 ml/min Oven temp. initial 70 °C-1 min. hold; 10 °C/min ramp to 160 °C hold 2 min 5 °C/min ramp to 210 °C; 5 min hold 3 °C/min ramp to 250 °C; 15 min hold
Software	X-Caliber	Chem station
Library	NIST	NIST

Table 2. Tryptophan metabolites and their mass spectral data.

S. no.	Compound	m/z	M. formula	%	MS fragmentation m/z
1	Serotonin	176	C ₁₀ H ₁₂ N ₂ O ₃	0.43	320-TMS 249-TMS 177,163,156,149
2	3-Indole lactic acid	205	C ₁₁ H ₁₁ NO ₃	0.71	277-TMS 206,187,169,156
3	5-hydroxytryptophan	220	C ₁₁ H ₁₂ N ₂ O ₃	1.53	292-TMS 221,263,207,193,179
4	5-(OH)-Indoleacetic acid	191	C ₁₀ H ₉ NO ₃	1.73	263-TMS 193,191,231,221,207,202,177
5	N-acetyl serotonin	218	C ₁₂ H ₁₄ N ₂ O ₂	0.35	300-TMS-219,285,219,201,201
6	2-Aminofumaric acid	213	C ₈ H ₇ NO ₄ S	2.88	285-TMS-213,209,208,194,192,191
7	Kynurenic acid	189	C ₁₀ H ₇ NO ₃	1.05	405-TMS 330-TMS 261,TMS 189,312,202,193,156
8	Indole acetic acid	175	C ₁₀ H ₉ NO ₂	1.01	319-TMS 248-TMS 175,168,156
9	3-(OH) kynurenine	224	C ₁₀ H ₁₂ N ₂ O ₄	1.34	296-TMS 225,266,210,197,207
10	Melatonin	232	C ₁₃ H ₁₆ N ₂ O	0.46	232,202,215,179,171,156
11	3-Indole carboxylate	161	C ₉ H ₉ NO ₂	0.42	377-TMS 304-TMS 233,161,156,147
12	Tryptophan (Trp)	204	C ₁₁ H ₁₂ N ₂ O ₂	6.49	202,204,344,266,130,73
13	Tryptophan ethyl ester	232	C ₁₃ H ₁₆ N ₂ O ₂	2.16	304-TMS 232,207,205,202
14	3-Indole pyruvate	203	C ₁₁ H ₉ NO ₃	0.43	491,203,341,339,267,231,202,191
15	Quinolinic acid	167	C ₇ H ₅ NO ₄	5.31	311-TMS 267-TMS 167,169,183,197
16	Nicotinic acid	123	C ₆ H ₅ NO ₂	2.87	339-TMS 269-TMS 197,123,
17	3-Indole acetamide	174	C ₁₀ H ₁₀ N ₂ O	0.18	318-TMS 245-TMS-174,258,185,156
18	o-aminohippurate	194	C ₉ H ₁₀ N ₂ O ₃	15.79	338-TMS 267-TMS 194,
19	Kynurenine (Kyn)	208	C ₁₀ H ₁₂ N ₂ O ₃	15.16	352-TMS 280-TMS,208 17,169,183,197
20	N-formyl kynurenine	236	C ₁₁ H ₁₂ N ₂ O ₄	12.02	380-TMS 308-TMS-238,225,211,197,183,169,
21	N-acetyl kynurenine	250	C ₁₂ H ₁₄ N ₂ O ₄	14.08	394-TMS 322-TMS-252,239,225,185

Mass spectral fragments of silylated compounds and their relative% yields are given in Table 2.

Time course studies showed that the formation of IAA could not be increased with an increase in incubation time, or a change in its media pH. Our results indicate that kynurenine pathway (KP) may be the major route for conversion of Trp to IAA as observed by Martens and Frankenberger who studied the metabolism of Trp,²⁸ where Trp is reported to be the primary precursor of IAA in soil^{39,40} and Trp transaminase is the major enzyme involved in its conversion into IAA.⁴¹ The enzyme, L-Trp aminotransferase (L-TAT) which is known to play a major role in the initial transformation of L-Trp into auxins in plant kingdom⁴¹ is an α -ketoglutarate-dependent conversion enzyme and indole-3-pyruvic acid is the product of this catalysis. In summary, for the first time, our work demonstrates that a single thermophile is responsible for transformation of L-Trp into IAA and many other metabolites.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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