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L-Phenylalanine and L-Tyrosine Catabolism by Thermophilic *Geobacillus stearothermophilus*

M. Afzal[1*], S. Al-Awadhi[1] and S. Oommen[1]

[1]Department of Biological Sciences, Faculty of Science, Kuwait University, Kuwait.

Authors' contributions

This work was carried out in collaboration between all authors. All authors contributed equally for this work. Author MA supervised the experimental work and wrote the manuscript. Author SA designed the experimental work and arranged the grant to carry out this work. Author SO carried out the experimental work. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: This study investigated the potential of soil thermophile *Geobacillus stearothermophilus* for the biotransformation of phenylalanine and tyrosine.
Study Design: *G. Stearothermopilus* grows well at 65ºC and has a good potential for transformation and biodegradation of many compounds including steroids, bile acids, tryptophan and other compounds. In this study *G. stearothermophilus* was harvested at mid-log phase at 65ºC, on tryptone yeast extract (TYE) medium. Cells were collected by centrifugation under aseptic conditions, washed with sterile water and suspended in phosphate buffer with phenylalanine or tyrosine as sole source of carbon at 65ºC. Metabolic parameters were optimized for optimal growth of the organism utilizing aromatic amino acids as an exclusive source of carbon.
Methodology: The amino acid metabolites were exhaustively extracted with methanol from freeze dried broth. The concentrated pooled extracts were analyzed by thin layer chromatography (TLC) using polar solvent systems and purification of the extracts was achieved on preparative tlc plates and GC separations. The molecular structures of purified metabolites were established through spectral data.
Results: Sixteen metabolites of phenylalainine and seventeen metabolites of tyrosine were identified in this study. Tyrosine metabolism extensively produced melanin pigments that caused hitches in the purification of tyrosine metabolites. Tyr metabolites were

**Corresponding author: Email: Afzalq8@gmail.com;*

analyzed in cells cultured for short time.
Conclusion: Our data suggest that *G. stearothermophilus* has a good potential to metabolize aromatic amino acids yielding hydroxylated, deaminated, decarboxylated and many other products. Oxidative metabolism of phenylalanine and tyrosine by a thermophilic *G. stearothermophilus* is being reported for the first time.

Keywords: Geobacillus stearothermophilus; aromatic amino acids; dopa, dopamine; transformation.

1. INTRODUCTION

Bacterial degradation of amino acids (AA) commonly takes place in the nature. Numerous soil bacteria also facilitate AA degradation by a number of mechanisms including oxidation and/or dehydrogenation processes [1]. Rumen bacteria are also known to degrade various AA resulting in various products [1]. Thus, a mesophile has been isolated from dairy waste water treatment plant, which could modify AA [2], and a *Pseudomonas* endowed with rich nutritional versatility has been used to study catabolism of a variety of AA [3]. Microbial degradation of AA can also be carried out by a variety of anaerobic saccharolytic and non-saccharolytic bacteria [4]. Many workers, for example, have studied microbial transformation of S-methylmethionine to homoserine [5-7].

The AA degradation can be catalyzed by transaminases and oxidases that cause oxidative deamination [8,9]. However, oxidases are neither ubiquitous nor essential to prokaryotic metabolism where D-stereomers of AA are degraded by D-amino acid dehydrogenases [3,10]. However, enzymes in soil bacteria, with their broad specificity, appear to have the faculty to metabolize both D- and L-amino acids [11]. Thus, degradation of both D- and L-phenylalanine (Phe) by soil bacteria has been reported [12]. It is known that prokaryotic D-amino acid deamination differs mechanistically from their eukaryotic counterparts resulting in the same end product. The resulting α-keto-acids may be decarboxylated yielding acyl-CoA derivatives and volatile ketones in the environment [13]. The use of AA to produce aroma compounds catalyzed by thermophilic lactic acid bacteria has been reported [14].

The mechanism of Phe degradation by *Escherichia coli* and *Pseudomonas putida* has been suggested *via* phenylacetate pathway [15]. The soil bacterial catalyzed deamination of Phe to give phenylacetate, benzoic acid and some other unknown products has also been reported [16-18].

Similarly, modification of phenolic AA such as tyrosine (Tyr) and other compounds such as flavones, hydroxyacetophenones by *Streptomyces peucetius* has also been demonstrated [19-21]. Phe/(Tyr) ammonia-lyase catalyzed deamination can give rise to cinnamic acids and their derivatives that act as important precursors in the biosynthesis of numerous phenolic compounds in plants [22]. While, bacterial metabolism of Phe and Tyr is known to give phenylpropanoids and other metabolites [23], the use of thermophilic *G. stearothermophilus,* for the degradation of phenylalanine and tyrosine to dihydroxyphenylalanine (DOPA) and many other metabolites, is being reported for the first time. Thermophilic enzymes have numerous advantages over the conventional enzymes for their ability to withstand elevated temperatures and resist organic solvents and recently, DNA polymerase from thermophilic bacteria has become an important tool in molecular biology for the development of super-thermophilic bacteria [24]. The aim of the work was to demonstrate the conceivable potential

of *G. stearothermophilus* to modify aromatic amino acids to a range of other small molecules.

2. MATERIALS AND METHODS

2.1 Organism

The thermophilic *Bacillus* strain used in this work was isolated from the oil contaminated desert soil of in Kuwait as previously described [25,26]. The isolate was a rod-shaped, Gram-positive bacterium identified as *Bacillus stearothermophilus* (Bacillaceae) that could grow at 75ºC and could survive on crude oil as an exclusive carbon source. Its identification was confirmed by the National Collection of Industrial and Marine Bacteria, Terry Research Station, Aberdeen, Scotland, U.K. *G. stearothermophilus* has the capacity to adapt to diverse nutritional requirements. The strain was maintained at 4ºC on tryptone and yeast extract agar plates as described previously [25]. Seed cultures, (50 mL/l) of *G. stearothermophilus* were grown on tryptone yeast extract (TYE) medium for 14hr in a shaking incubator at 65ºC using aerobic conditions. The seed cultures were transferred to fresh 500 mL TYE media in 2L baffled flasks and were shaken at 65ºC until mid-log phase of growth (3.5 - 4hr). The cells were aseptically collected by centrifugation at 8000rpm for 20min followed by their suspension in sterilized 5-10mL of 0.05M phosphate buffer (pH 7). The suspension was transferred to two Erlenmyer flasks (500mL capacity) containing 200mL of the 0.05M sterile phosphate buffers pH 7 & pH 5 and 10% of Castenholz mineral salt solution as described by us previously [26]. An aqueous solution of Tyr/Phe was added to achieve a final concentration of 1mg mL^{-1} and incubated at 65^{o}C for a period of 72 hr. Standard Tyr and Phe in phosphate buffer pH 5 & 7 without the addition of cells were also examined to check their aerial oxidation in the buffer. However, no degradation was observed in these experiments confirming that all Tyr and Phe products were bacterial catalyzed modification products.

2.2 Extraction and Isolation of Transformed Products

The incubated cultures were freeze-dried after adjusting the pH to 6.8 and the freeze-dried powder was extracted with acetonitrile by stirring overnight at 40ºC followed by an extraction with acetonitrile:water (70:30, v/v). After filtration, the organic solvent from the pooled extract was removed on a rotary evaporator while the remaining water was removed by freeze-drying. The material thus obtained was re-dissolved in acetonitrile:water (70:30, v/v) and chromatographed on preparative thin layer chromatography (TLC) plates 20×20cm, 500µm thickness (Sigma Aldrich, #Z512990) using benzene:ethylacetate:acetone;methanol:water (22:20:3:3:2, v/v) and also butanol:acetic acid:water (2:1:1,v/v) as mobile phases. Well resolved bands, as visualized under UV light (λ 254nm) were marked by a pencil. A two inch side strip of the chromatogram, while covering the rest of the plate with a glass sheet, was stained with ninhydrin solution prepared in aqueous acetone. The chromatogram, after heating at 110ºC for 10 min., developed colored bands corresponding to amino acids. Corresponding bands from the TLC plates were scratched, pooled, re-extracted into acetonitrile:water (70:30, v/v) and concentrated under vacuum. The concentrated extract was re-chromatographed on TLC plates until pure compounds were obtained.

2.3 Quantification and Derivitization of Metabolites

2.3.1 Spectroscopic methods

^{1}H and ^{13}CNMR spectral data were used when sufficient material was collected from tlc purification procedure. The data were collected for samples dissolved in d_4-methanol, using a 600 MHz NMR (Bruker AC 600) instrument. Mass spectral data were obtained using GC/MS Agilent Technologies instrument model MSD 5973 attached with an Agilent autosampler model 7683.An OV-1 capillary column with 25m length and 0.35µm od was used with this instrument. GC/MS spectra were also determined using GC/MS DFS Thermo Finnigan TOF instrument interfaced with NIST library data base. The ionization mode was EI at 70ev, with probe heating from 50-250ºC at 6ºC min^{-1}. A DB5 column 30m x 0.25mm x 0.25 µm was used with this instrument. A split injection 1:1 was used for GC analyses and accurate mass measurements were made to calculate the molecular formulae of the compounds. MS/MS was used for identification of the molecular structures of the metabolites.

For GC analyses of amino acids, a pre-column derivatization of amino acids was carried out with phenyl isothiocyanate (PITC) according to the recommended procedure of Pico-Tag [27]. A second derivatization of amino acids was achieved with BSA, BSTFA and BSTFA+TMSC. For derivatized samples, the GC heating program was initially programmed at 70-250ºC at 10ºC min^{-1} to 160ºC followed by 5ºC min^{-1} ramp till 210ºC, followed by 3ºC min^{-1} ramp to a final temperature 250ºC. Identification of the metabolites was carried out by a direct comparison of the MS fragmentation with the standards run under identical conditions.

3. RESULTS AND DISCUSSION

The aromatic amino acids L-Phe and L-Tyr were degraded by a thermophilic bacterium *G. stearothermophilus* at 65ºC yielding DOPA and many other metabolites. TLC analyses of the *G. stearothermophilus* catalyzed transformation products of these amino acids indicated that 72 hrs incubation time was optimum for Phe-degradation to furnish its modified products in reasonable yields. However, Tyr incubation for 72 hr with *G. stearothermophilus* resulted in a dark brown-pigmented tarry mixture which resisted isolation and purification of Tyr metabolites. For this reason, Tyr incubation with *G. stearothermophilus* was carried out only for 24 hr when most of the amino acid had been consumed. Under these experimental conditions, from a total of sixteen metabolites of Phe, as observed on the TLC, eleven could be separated and identified without derivatization. The metabolites identification was carried out from their accurate mass measurement, mass spectral (MS) fragmentation and MS-MS analyses (Table 1). While after phenylisothiocyanate (PITC) derivatization of the Phe transformation crude mixture, three amino acids, Phe, Tyr, DOPA were tentatively confirmed. These amino acids were not visible in the GC chromatogram before derivatization. All transformed products of Phe, before and after derivatization, were effectively separated on a DB5 column using both GC/MS instruments (Figs. 1, 2).

Table 1. Phenylalanine metabolites before derivatization. Retention time (RT), molecular ion (m/z) and relative % concentration

Peak number	RT	Metabolite	m/z	% PA
1	5.55	Benzaldehyde	106.1223	1.45
2	6.78	Phenylethylamine	121.1792	82.48
3	8.83	Phenylacetaldehyde	120.1485	0.88
4	10.18	Phenylacetic acid	166.1740	3.99
5	12.1	Cinnamic acid	148.1583	0.29
6	12.45	Homovanillic acid	182.1733	0.52
7	15.66	Succinic acid	118.0873	6.64
8	16.05	Phenylpyruvic acid	164.1584	1.97
9	17.02	Protocatechuic acid	154.1197	0.68
10	18.16	Cinnamaldehyde	132.1595	0.44
11	18.33	Homogentisic acid	168.1478	0.66

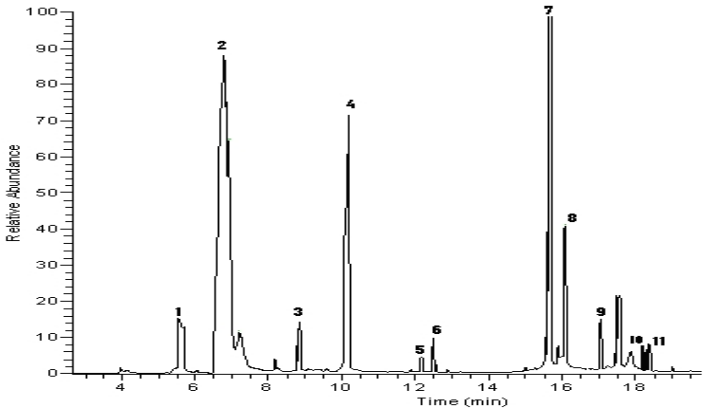

Fig. 1. GC separation of Phenylalanine metabolite before derivatization
1. Benzaldehyde; 2. phenylethylamine; 3. phenylacetaldehyde; 4. phenylacetic acid; 5. cinnamic acid; 6. homovinillic acid; 7. succinic acid; 8. phenylpyruvic acid; 9. protocatechuic acid; 10. cinnamaldehyde; 11. homogentisic acid

The formation of these products could be envisioned through hydroxylation, Phe/Tyr/DOPA, deamination to cinnamic acid derivatives, and decarboxylation to dopamine. This indicated the presence of phenylalanine hydroxylase and DOPA oxidase p-450 activities in *G. stearothermophilus*. Bacterial phenylalanine hydroxylase and DOPA oxidase activities have been demonstrated in Pseudomonas sp [28]. The Phe and Tyr conversions may be facilitated by specific cytochrome P-450, mixed function mono-oxygenases and ammonia lyases commonly present in Sterptomyces and reported by many workers [29-32].

Tyr metabolism by *G. stearothermophilus* added complexity to the transformation products due to melanin formation that was apparent after short incubation of the amino acid [33]. Extended incubation time of Tyr with the bacterium, resulted in an increased formation of the pigment, presumably due to the formation of polymeric homogentisic acid and DOPA followed by melanin development [34]. Therefore, incubation time of the amino acid with the bacterium, was restricted to 24 hrs when most of the amino acid was consumed yielding degradation products with minimal pigment formation. Microbial degradation of Tyr resulted in seventeen products, identified through their MS fragmentation and MA-MS analyses (Fig.

2, Table 2) suggesting that this amino acid could be a better enzyme substrate for its modification. All tyrosine metabolites were effectively separated on a capillary GC-DB5 column with or without derivatization (Fig. 2).

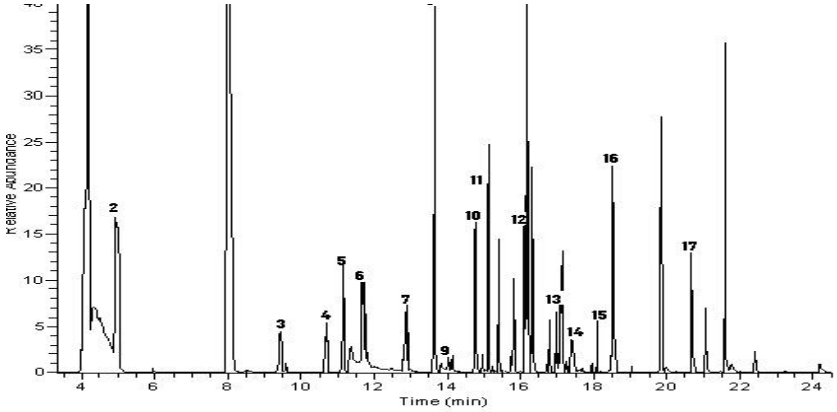

Fig. 2 Tyrosine degradation catalyzed by *G. stearothermophilus*. GC/MS without derivatization

1. 4-Hydroxyphenylacetate; 2. Adipic acid; 3. Succinic acid; 4. 4-Hydroxybenzaldehyde; 5. Phenylalanine; 6. 4-Hydroxybenzoic acid; 7. 3, 4-Dihydroxyphenyl acetate; 8. Tyrosine; 9. 4-hydroxyphenylacetic acid; 10. Homogentisic acid, 11. 2, 4-dihydroxyphenyl acetic acid; 12. Cinnamic acid; 13. Prtocatechuic acid; 14. Homovanillic acid; 15. 4-hydroxyphenyl pyruvate; 16. Phenylacetaldehyde; 17. DOPA

Table 2. Tyrosine metabolites, their retention times (RT), molecular ion and relative % concentration

Peak number	RT	Compound	m/z	% PA
1	4.15	4-Hydroxyphenylacetate	152.1470	9.85
2	4.99	Adipic acid	146.1413	7.03
3	9.39	Succinic acid	118.0877	2.79
4	10.58	4-Hydroxybenzaldehyde	122.0372	2.47
5	11.17	4-Hydroxyphenylethylamine	165.1891	4.33
6	11.6	4-Hydroxybenzoic acid	138.1214	5.19
7	12.87	3, 4-Dihydroxyphenyl acetate	168.1468	5.07
8	13.65	Tyrosine	181.1883	22.6
9	13.81	4-hydroxyphenylacetic acid	152.1472	5.36
10	14.75	Homogentisic acid	168.0423	9.49
11	15.16	2, 4-dihydroxyphenylacetic acid	168.1468	2.25
12	16.00	Cinnamic acid	148.1587	2.07
13	17.12	Prtocatechuicacid	154.1198	6.50
14	17.39	Homovanillic acid	182.1731	1.21
15	18.10	4-hydroxyphenyl pyruvate	180.1574	1.14
16	18.51	Phenylacetaldehyde	120.1485	7.09
17	20.66	DOPA	197.1882	5.46

Typically, *Streptomyces* species are known to utilize tyrosine for the formation of DOPA thus resulting in melanin formation [35,36]. *S. coelicolor* possesses a DOPA decarboxylase that could convert DOPA to dopamine. In plants, 4-hydroxyphenylpyruvate dioxygenase (4HPPD, EC 1.13.11.27) is a key enzyme in the biosynthesis of plastoquinones and tocopherols. 4-Hydroxyphenylpyruvate (derived from chorismic acid via the shikimate pathway) is oxidized and decarboxylated by 4HPPD to yield homogentisic acid, a melanin precursor [37-39]. In Tyr oxidative degradation pathway, 4HPPD could catalyze the conversion of homogentisic acid (HGA) to melanin pigment. An expression of 4HPPD family members is known to result in the accumulation of HGA-melanin by tyrosine-tyrosinase reaction under aerobic conditions [40]. Hydroxylation of the phenyl ring to yield hydroxyaromatic acids with concomitant deamination of the amino acid followed by oxidation, could take place by monooxygenases, cytochrome P450 CYP153 family [41] and 4HPPD family of enzymes [40] and transaminases, commonly present in soil bacteria [42,43]. Thus longer incubation period of Tyr with *G. stearothermophilus* makes it difficult to study its microbial degradation. Melanins are known by products of spontaneous reactions involved in oxidative polymerization and copolymerization of DOPA, homogentisic acid, and p-polyhydroxy-, (poly)hydroxy(poly)amino- and polyamino-phenyl products [44].

Bacterial catalyzed decarboxylation/deamination commonly takes place resulting in tyramine poisoning generated from tyrosine decarboxylation [6,45-47]. Thus, a decarboxylation of the corresponding amino acid resulted in the formation of phenylethylamine which could undergo oxidative deamination catalyzed by ubiquitous bacterial amine oxidases resulting in phenylpyruvate and its phenolic derivatives through hydroxylation of the aromatic ring [48]. Bacterial strains of *enterobacteriaceae* family such as *Klebsiella, Enterobacter, Eschericia, Salmonells, Serratia* and *Proteus*are are shown to carry such amine oxidases [49]. Tyr has been investigated as a nitrogen sources encoding for homogentisate dioxygenase (*hmgA*) gene, involved in Tyr catabolism [50,51].

In the present study, we have observed the hydroxylation of phenylalanine resulting in tyrosine. But at the same time, we observed an inhibition of bacterial growth that may be due to the possible formation of m-tyrosine which is a known inhibitor for plant and bacterial growth [52]. However, the presence of m-tyrosine could not be tentatively confirmed from the MS analyses of the metabolites.

The formation of cinnamaldehyde could be explained via cinnamate dehydrogenase action on cinnamic acid while formation of 4-hydroxyphenylacetic acid could be either from Tyr after oxidative decarboxylation of 4-hydroxyphenylpyruvate, leading to 4-hydroxybenzoate and protocatechuate. Similarly, phenylacetaldehyde may appear from phenylacetic acid, a metabolite of Phe. Although degradation of Phe and Tyr have been studied before in soil bacteria where synergistic action from other bacteria may play an important role, our study was carried out using an isolated bacterium that eliminated the possibility of bacterial synergism. Therefore, all components reported in this study are solely biodegradation products of Phe and Tyr catalyzed by *G. stearothermophilus*. This study emphasizes the fact that *G. sterothermophilus* when offered the right growth conditions, has excellent potential for biotransformation of a diverse class of compounds such as lipophilic steroids and ionic amino acids.

4. CONCLUSION

Data show that *Geobacillus stearothermophilus* has an excellent potential to transform Phe and Tyr into many products that could be separated and identified by GC/MS using a DB5 capillary column. The thermophilic bacterial catalyzed formation of DOPA from Phe and Tyr is demonstrated for the first time.

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COMPETING INTERESTS

No competing interests are declared by all authors.

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