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DAHP synthase of phenylalanine non-producing and phenylalanine producing mutant strains of *Arthrobacter globiformis*

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3-Deoxy-D-arabinoheptulosonate 7-P (DAHP) synthase from wild type, tryptophan and tyrosine double auxotrophic mutant and double auxotrophic multianalogue resistant mutant of *Arthrobacter globiformis* were assayed. Maximum enzyme activity was observed in late exponential phase and TT-39 PTMN^r-7 retained maximum activity at late stationary phase of growth. Tryptophan, tyrosine and phenylalanine repressed the enzyme synergistically but TT-39 PTMN^r-7 was less sensitive to repression. Tryptophan, tyrosine and phenylalanine synergistically inhibited the enzyme but on their own did not produce sufficient inhibition.

BIOSYNTHESIS of aromatic amino acids begins with condensation of phosphoenolpyruvate and erythrose 4P by the enzyme DAHP synthase (EC. 4.1.2.15). This is the main feedback control point of the branched pathway and the aromatic amino acids control the activity of this enzyme. Micro-organisms show considerable diversity in the feedback regulation of DAHP synthase[1–4]. In *E. coli*[5–7], *Neurospora crassa*[8], *Salmonella typhimurium*[9], *Claviceps paspali*[10] and *Saccharomyces cerevisae*[11, 12], the reaction was carried out by three isozymes, the activity of which are inhibited by L-tyrosine, L-phenylalanine and L-tryptophan respectively. In *Bacillus subtilis*[2, 13] chorismate and prephenate (branched point intermediate) inhibited DAHP synthase whereas aromatic amino acids did not. Other control patterns reported are cumulative feedback inhibition found in *Hydrogenomonas*[2] sp, concerted feedback inhibition found in *Rhodomicrobium vanneili*[14], unimetabolite control found in *Pseudomonas*[2] sp where only tyrosine and in *Streptomyces aureofaciens*[15] where only L-tryptophan inhibited the enzyme. Synergistic inhibition has been reported in *Brevibacterium flavum*[16] and *Corynebacterium glutamicum*[17]. By mutagenesis with NTG, the present authors were able to obtain both double auxotrophic mutants[18,19] and double auxotrophic multianalogue-resistant mutants of *Arthrobacter globiformis*. Both these mutants produced L-phenylalanine which is an essential amino acid for human nutrition, now widely used as constituent for the dipeptide sweetener 'Aspartame'[20].

The present paper reports the activity of DAHP synthase under different phases of growth in wild type (A8), double auxotrophic mutant (TT-39) and double auxotrophic multianalogue resistant mutant (TT-39PTMN^r-7) of *Arthrobacter globiformis*. The pattern of regulation of the DAHP synthase of different strains has also been compared.

A. globiformis (A8), a glutamate producer, was isolated from Burdwan soil[21]. The tryptophan and tyrosine double auxotroph (TT-39) and double auxotrophic multianalogue-resistant mutant of TT-39PTMN^r-7 of *A. globiformis* were isolated by mutagenesis with NTG treatment[19]. The strains were maintained on Alfoldis[22] agar slant with supplements as required. The organisms were grown in shaken flask using the same liquid medium on a rotary shaker at 30°C. Growth was measured turbidimetrically and extra-cellular phenylalanine estimated by paper chromatography and micro-biological assay[18, 19]. An aqueous suspension of bacteria from a 24 h old culture on agar slant (10^6 cells ml^{-1}, 2% v/v) was to inoculate the flasks. The cells were harvested by centrifugation of 1000 *g* for 45 min in cold centrifuge and washed twice with 0.04 M cold potassium phosphate buffer (pH 7.0). Washed cells were resuspended in the above buffer at a cell density of 500 mg of fresh weight per ml and disrupted by sonication for 4 min on an ice bath using ultrasonic disintegrator (Soniprep-20) and the extract was centrifuged at 10000 g for 20 min at 2°C. The extract was dialysed overnight using the same buffer and its protein content was measured[23]. The DAHP synthase was assayed[24] by measuring the absorbance at 549 nm (the β-formyl-pyruvic acid formed) with UV-visible double beam spectrophotometer (Shimadzu).

The activity of DAHP synthase in wild type (A8), double auxotroph (TT-39) and double auxotrophic multianalogue-resistant mutant (TT-39 PTMN^r-7) increased gradually with growth (Table 1). In all the three types (A8, TT-39 and TT-39 PTMN^r-7), the DAHP synthase activity was maximum at late exponential phase of growth. The enzyme activity of A8 and TT-39 declined 40% and 25% of the maximal activity respectively during the late stationary phase of growth but TT-39PTMN^r-7 retained about 90% of the maximal activity at this stage. TT-39 and TT-39PTMN^r-7 showed 60% and 200% greater activity of the DAHP synthase respectively over the phenylalanine non-producing *A. globiformis* (A8). Shetty *et al.*[25] reported a similar increase of DAHP synthase activity when the cells of phenylalanine producing *Bacillus polymyxa* approach the stationary

phase of growth.

The pattern of regulation of DAHP synthase of the three strains was compared by adding tryptophan and tyrosine in different levels. These levels were critical for phenylalanine production in TT-39 strain of *A. globiformis*[19]. For phenylalanine production tryptophan and tyrosine were found to be optimum at 0.5 mM and 0.1 mM levels respectively. Cells were cultivated in optimized medium with supplementation of different levels of tryptophan and tyrosine and harvested at the end of the exponential phase of growth and assayed as earlier.

The results (Table 2) revealed that tryptophan (5 mM) and tyrosine (1 mM) showed a synergistic repression of 30%, 28% and 12% in A8, TT-39 and TT-39 PTMN^r-7 respectively. The synergistic repression was further increased to 40%, 35% and 20% respectively when phenylalanine (1 mM) was added in addition to tryptophan (5 mM) and tyrosine (1 mM). Tryptophan at higher concentration in comparison to phenylalanine and tyrosine was required to produce repression in any of the strains. The enzyme of double auxotrophic multianalogue-resistant mutant was less sensitive to repression in comparison to double auxotroph or wild type. Repression of the DAHP synthase by phenylalanine, tyrosine and tryptophan has been reported in *E. coli*[26]. But in *Bacillus subtilis*[27] and *Brevibacterium flavum*[28] the enzyme was repressed by only tyrosine. On the other hand the enzyme of *Nocardia*[29] sp 239 is not repressed by any of the aromatic amino acids.

Addition of tryptophan, tyrosine and phenylalanine individually or in combination with the assay mixture revealed (Table 3) that in none of the strains tryptophan at 5 mM level inhibits the enzyme activity. But inhibition was observed by tyrosine or phenylalanine. Addition of tryptophan with either tyrosine or phenylalanine did not increase inhibition in any of the three strains studied but when tyrosine and phenylalanine were together in the assay mixture, a synergistic inhibition of the enzyme activity was noted in all the strains. Synergistic inhibition was more pronounced in A8, TT-39 and TT-39PTMN^r-7, when tryptophan, tyrosine and phenylalanine were added (5 mM each) together in the assay mixture. Similar inhibition of DAHP synthase with aromatic amino acids has been observed in *Corynebacterium glutamicum*[17]. The double auxotrophic multianalogue-resistant TT-39PTMN^r-7 showed higher activity of DAHP synthase which was less sensitive to inhibition by aromatic amino acid(s). A similar high specific activity of DAHP synthase in analogue-resistant mutant strain of *B. polymyxa* as compared with the parent was reported by Shetty *et al.*[25]. They concluded that analogue-resistant mutant either partially or wholly depressed the DAHP synthase. The increased activity of DAHP synthase is probably one of the reasons for overproduction of L-phenylalanine by this mutant. Similar deregulation of DAHP synthase was observed in β-2-thienylalanine (analogue of phenylalanine)-resistant phenylalanine hyperproducing mutant of *E. coli*[30,31] and *Bacillus subtilis*[32].

The increase in phenylalanine production by TT-39 PTMN^r-7 observed in the present investigation may be assigned to (i) increased specific activity of DAHP synthase over the parent strain (ii) relative insensitivity to repression or inhibition by phenylalanine accumulated alone in the culture medium and (iii) persistence of a good portion of its maximal activity during the post-logarithmic phase of growth. Thus, even a higher level

Table 1. DAHP synthase activity during different phases of growth in A8, TT-39 and TT-39PTMN^r-7

	A8		TT-39			TT-39PTMN^r-7		
Time (h)	Gr[a]	DAHP synthase activity[b]	Gr[a]	DAHP synthase activity[b]	Phe[c]	Gr[a]	DAHP synthase activity[b]	Phe[c]
12	2 1	6	1 8	10	0 3	1 5	15	0 7
24	6 1	8	5 7	12	1 8	5 5	20	2 9
36	9 9	10	9 1	16	3 0	8 4	30	4 2
48	10 5	8	9 9	16	4 1	9 0	30	6 5
60	9 8	7	9 0	15	5 0	8 1	28	7 6
72	9 8	6	9 0	12	6 8	8 1	28	9 6

[a] = Growth, O D in EEL Unit; [b] = Units, n mol of DAHP formed min^{-1} mg^{-1}; [c] = Phenylalanine g/l

A8 = Wildtype of *A globiformis* (phenylalanine non-producer).

TT-39 = Tryptophan and tyrosine double auxotroph of *A globiformis* (phenylanine producer)

TT-39PTMN^r-7 = Tryptophan plus tyrosine double auxotroph and p-fluorophenylalanine (phenylalanine analogue), β-2 thienylalanine (phenylalanine analogue), 5-methyl-tryptophan (tryptophan analogue) and 3-Nitrotyrosine (tyrosine analogue) resistant mutant of *A. globiformis* (phenylalanine producer)

Table 2. Repression of DAHP synthase in presence of aromatic amino acids (different level) added in the growth medium

Amino acid (mM) added in growth medium			Repression (%)		
L-Trypto-phan	L-Tyro-sine	L-Phenyl-alanine	A8	TT-39	TT-39PTMN^r-7
0 5	0 1	–	0	0	0
0 5	1.0	–	20	20	8
5 0	0 1	–	5	5	2
5 0	1 0	–	30	28	12
5.0	1 0	1 0	40	35	20

Table 3. Feedback inhibition of DAHP synthase in the presence of aromatic amino acids (different levels) added in the assay mixture

Amino acids added in assay mixture	Inhibition (%)		
	A8	TT-39	TT-39PTMN^r-7
Control (Absence of amino acids)	0	0	0
L-Trp (5 mM)	0	0	0
L-Tyr (5 mM)	20	20	5
L-Phe (5 mM)	22	22	8
L-Trp + L-Tyr (5 mM each)	20	20	5
L-Trp + L-Phe (5 mM each)	22	22	8
L-Tyr + L-Phe (5 mM each)	75	72	32
L-Tyr + L-Phe + L-Trp (5 mM each)	98	90	52

L-Tyr = L-Tyrosine; L-Trp = L-Tryptophan; L-Phe = L-Phenylalanine.

of intra-cellular phenylalanine under this condition cannot make the synergistic inhibition of DAHP synthase operative as tyrosine and tryptophan level in the medium for these double auxotrophs are maintained at a level far below the required level.

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Observations on fungal infection of *Chela laubuca* Ham. with special reference to deep mycoses

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Some specimens of *Chela laubuca* Ham., bearing fungal infection on the body surface and eye, were collected from Domingarh Pond and Garden Water Tank of the Office of N.E. Railway, Gorakhpur, UP, India. The causal watermoulds have been identified as *Achlya orion* Coker & Couch, *Saprolegnia diclina* Humphrey, *S. ferax* (Gruith.) Thuret and *Pythium aphanidermatum* (Ed.) Fitz. Histopathological studies of infected skin have shown a varying degree of destruction of epidermis, hypodermis and the underlying musculature whereas infected eye has shown profuse hyphal growth, inflammation of cornea, disintegrated iris and deshaped retina. The pathogenic nature of the fungal isolates has also been proved under laboratory conditions.

THE pioneering work in the field of fish-mycopathology in India is that of Gopalakrishnan[1] who described fish mycoses caused by *Saprolegnia parasitica* in Indian waters. Subsequently, many other workers reported numerous watermould species parasitizing different species of fish and their eggs[2–11]. However, a perusal of the literature indicates very few reports on the deep mycoses in fish, in India[9,11].

During the course of investigations on fungi associated with fish diseases some specimens of *Chela laubuca* Ham., showing hyphal tufts protruding out through eyes and body surface, were collected during November 1982 to February 1983 and October 1990 to January 1991 from Domingarh Pond and Garden water tank of the Office of N.E. Railway, Gorakhpur, U.P. The infected living and dead specimens of fish were collected using hand-nets and brought to the laboratory in large-sized polythene bags, half-filled with fresh water. The infected fish, when placed in clean water, showed white cottony patches with hyphal tufts on body surface and eyes (Figure 1). Small bits of mycelium were taken out from white cottony patches and rinsed thoroughly in distilled water and were then placed in petri-dishes containing 10 ml of sterile distilled water on boiled hemp-seed cotyledons. Unifungal, bacteria-free cultures of the fungi were raised on the lines described earlier[12–14]. The fungi were identified as *Achlya orion* Coker & Couch (November 1982, October 1990; from body surface), *Saprolegnia diclina* Humphrey (December 1982, December 1990; from eye and body surface), *Saprolegnia ferax* (Gruith.)