CHAPTER IV

NITROGEN METABOLISM AND ALKALOID FORMATION DURING SHAKE CULTIVATION OF Claviceps species Strain SD-58
Nitrogen is an essential component of all living systems. Accordingly, a significant proportion of metabolic activities of an organism is centered around the procurement and assimilation of nitrogen. It has been demonstrated in variety of micro-organisms that inorganic nitrogen may be assimilated into amino nitrogen via glutamate, alanine, aspartate, carbamyl phosphate and glutamine, but no organism utilizes all these routes with same degree (Moat and Ahmed, 1965; Nicholas, 1965). Usually, a given organism will utilize one or two pathways predominantly to the virtual exclusion of the other (Helperl and Umberger, 1960; Pateman and Kinghorn, 1965; Desai and Modi, 1977). Although, considerable amount of work has been done on the biochemistry and genetics, of nitrogen
metabolism in various prokaryotes (Magasanik, 1982; Walls et al., 1983; Ali et al., 1981; Merrick, 1982; Fischer and Sonenschein, 1984), majority of investigations in eukaryotic systems has been done in only three organisms: Neurospora crassa, Aspergillus nidulans and Saccharomyces cerevisiae. (Cove, 1979; Davis, 1975; Metzenberg, 1979; Pateman and Kinghorn, 1975, 1977; Schmit and Brody, 1976; Morluf, 1981). Certain compounds particularly ammonia, glutamate and glutamine, are found to be favoured nitrogen sources by fungi. However, capability of utilizing many diverse secondary nitrogen sources including nitrate, nitrite, purines, proteins, numerous amino acids, acetamide and even acrylamide by fungi have been reported (Murzluf, 1981; Pateman and Kinghorn, 1975).

Numerous investigations on the effect of various nitrogen sources on the production of secondary metabolites have been reported (Martin and Demain, 1980; Aharonowitz, 1980; Turner and Aldridge, 1982). Some amino acids or nitrogen compounds appear to be stimulatory due to their role as triggerers of differentiation rather than as precursors. The role of methionine in cephalosporine production by Cephalosporium acremonium (Martin, 1978) of benzylthiocynate in chlorotetracyclin production by Streptomyces aureofaciens (Hostalek et al., 1979) and of
kanamycin in aerial mycelium formation by *Streptomyces alboniger* (Pogell, 1979) are some of the examples. Ammonium ions (organic/or inorganic) has been found to be an essential ingredient of the cultivation medium for many micro-organisms. In the last two decades, various investigators have concerned themselves to investigate the regulatory role of ammonium nitrogen, particularly with respect to secondary processes and this has been a subject of many reviews (Turner and Aldridge, 1982; Nowacki *et al.*, 1975, 1976; Martin, 1978).

Though, ammonium ion is an essential elements for the production of secondary metabolites the amount needed for the optimum production varies considerably. The production of antibiotics like streptomycin by *Streptomyces griseus* (Nova *et al.*, 1983), gramicidin-s by *Bacillus brevis* (David and Demain, 1984) and Penicillin by *Penicillium crysogenum* (Motoes *et al.*, 1984) have been found in the concentration range of 1.0 to 1.7 N, while for vitamins like Vitamin K$_2$ by *Flavobacterium meningoseptium* (Tani *et al.*, 1984) and B$_{12}$ by *Propionibacterium freudenreichii* (Yongsmit and Apiraktivongse, 1984) produced at very lower ammonium concentration (0.15 to 0.5 M). The requirement of nitrogen for alkaloid biogenesis by *Claviceps* sps. have been reported to be 2.12 N (Rehacek *et al.*, 1977). Several investigations on the effect of various nitrogen sources
on the yield of ergot alkaloid have been performed (Banks et al, 1974; Bu'Lock, 1975; Robbers et al, 1978; Fornal, 1979). Kozlovskii et al, (1982) observed that addition of L-histidin, DL-mevalonic acid and L-tryptophan in fermentation medium caused induction in alkaloid production by Penicillium requefortii. Similarly, Robbers et al, (1982) observed 3.5 fold induction in alkaloid production over the control, when culture was provided with DL-B 1, DL-B 2 and L-B 1 - nepthalanline as nitrogen source. Banks et al, (1974) observed two fold increase in alkaloid production on addition of asparagine as sole nitrogen source in fermentation medium of C.fusiformis. However, our understanding on the nitrogen effect at the cellular level on alkaloid biosynthesis remains obscure. While studying the factors affecting the higher production of alkaloids during the submerged cultivation of Claviceps spp. Strain SD-58 (Chapter III), it was found that when asparagine was supplemented as the nitrogen source caused significant stimulation in alkaloid yield.

In view of the above it was interesting to investigate the role of nitrogen source on the physiology of alkaloid production during the submerged cultivation of Claviceps sp., Strain SD-58. Studies were initiated
Fig. IV. 1: Effect of nitrogen source on alkaloid production (a) and growth (b) of *Claviceps* sps., Strain SD-58. From the NL-406 medium, nitrogen source was omitted and desired nitrogen sources e.g. asparagine (o), aspartic acid (■), ammonium nitrate (■) and aspartic acid + ammonium nitrate as 1:1 ratio (●) was incorporated.
by cultivating *Claviceps* sps. Strain SD-58 in NL-406 medium devoid of nitrogen source and supplementing the desired nitrogen source as 4.12 N. Figure IV.1 illustrates the effect of nitrogen source on alkaloid production and growth of *Claviceps* sps. Strain SD-58. Asparagine showed the maximum stimulatory effect on alkaloid production among the nitrogen sources tested. Ammonium salts were found to be poor nitrogen source. When inorganic nitrogen source (ammonium salts) were partially or totally replaced by other organic nitrogen sources, i.e. asparagine, aspartate or glutamate about 1.5 to 3 fold increase in alkaloid yield and substantial increase in mycelial dry weight was observed. Though, different nitrogen sources gave different alkaloid yields, the fermentation cycle and growth cycle were not found to be affected and maximum alkaloid production was attained on 12 days of cultivation. Thus, it is reasonable to believe that the nitrogen source affects the rate of alkaloid synthesis. Our results are in line with those of reported by Marzlot (1981), Samchez et al., (1981) and Inova et al.,(1982), for production of antibiotics. Rehacek et al.,(1977) have observed similar effect in *C.purpurea* (Fr.) Tul.

Asparaginase is the first enzyme responsible for degradation of asparagine, and convert it to aspartic acid and ammonia. The activity of asparaginase, alkaloid
Fig. IV. 2: Specific activity of asparaginase (o) Asparagine synthetase (Δ), Intracellular ammonium ion concentration (x) and alkaloid content (t) during the submerged cultivation of Claviceps sps., Strain SD-58.
production and ammonium ion concentration during the submerged cultivation of *Claviceps* sps. Strain SD-58 is illustrated in Fig. IV-2. The activity of asparaginase increased up to the end of exponential growth period (8 day) and later fell during which alkaloid synthesis was found to be intensive. In general asparagine has been found to be taken up by micro-organisms without it being degraded before the entry as asparaginase has been found to be localized intercellularly. The fall in asparaginase activity after 8 day may be due to the sensitivity of asparaginase towards alkaloid as significant increase in the concentration of alkaloid is observed in this phase. The intracellular level of ammonia was found to parallel the activity of asparaginase. The rise in intracellular level of ammonia indicate its second preference over aspartic acid which is also a degradative product of aspartic acid by asparaginase. It was interesting to note the rapid utilization of intracellular ammonia during the intensive alkaloid producing phase of the growth. The level of asparaginase synthetase was found to remain at much lower than asparaginase and almost constant throughout the fermentation cycle (Fig.IV.2).

As the intracellular concentration of ammonia has been found to play an important role in biogenesis of many
Fig. IV. 3: The level of NADP⁺ glutamine dehydrogenase (•) and NAD-glutamine dehydrogenase (○) during the submerged cultivation of Claviceps sps., Strain SD-56.
secondary metabolites including alkaloid (Piorier and Demain, 1981; Maier et al., 1981; Nimi et al., 1982) and the drastic fall in intracellular level ammonium is associated with the intensive alkaloid production (Fig.IV.2), it was reasonable to investigate the assimilation of ammonia during alkaloid production by *Claviceps* sp. Strain SD-58.

In general, assimilation of ammonia has been reported by its incorporation into amino acid via several routes. However, only one or two routes have been found to be operated in any given organism with the exclusion of others. The activity of NAD$^+$ and NADP$^+$ linked glutamate dehydrogenase (GDH), the key enzyme linking glucose metabolism with amino acid biogenesis during shake cultivation of *Claviceps* species. Strain SD-58 is depicted in Fig.IV.3. The level of NAD$^+$-GDH was found to be comparatively lower than that of NADP$^+$-GDH. The activity of NADP$^+$-GDH remained at higher level in early period of growth cycle and gradually declined upto the 8 days of growth and after that increased marginally. It was interesting to note that activity of NADP$^+$-GDH decrease with the intensive alkaloid producing phase which is associated with the reduction in intracellular level of ammonium (Fig.IV.2). The inverse relationship between NADP$^+$-GDH and intracellular level of ammonia, rules out the major route of ammonia assimilation through
Table IV.1: Specific activities of NADP⁺-alanine dehydrogenase, NAD⁺-alanine dehydrogenase and glutamate synthetase during the submerged cultivation of *Claviceps* sps., Strain SD-58, grown in asparagine containing medium.

<table>
<thead>
<tr>
<th>Growth period (Days)</th>
<th>Sp. activity</th>
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<tr>
<td></td>
<td>NADP⁺ alanine dehydrogenase</td>
<td>NAD⁺ alanine dehydrogenase</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0.4</td>
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<tr>
<td>6</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>1.3</td>
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<tr>
<td>10</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>12</td>
<td>2.0</td>
<td>0.8</td>
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Fig. IV. 4: Specific activity of glutamine synthetase (○), and intracellular concentrations tryptophan (●) and ammonia (×) during the shake cultivation of Claviceps sps. Strain SD-58
The level of glutamate synthase, and alanine dehydrogenase enzymes have been listed in Table IV.1. The level of NADP⁺-alanine dehydrogenase and NAD⁺-alanine dehydrogenase and glutamate synthase are found to be very low as compared to other ammonia assimilating enzymes. Moreover, levels of these enzymes were not much altered during the entire fermentation cycle. Several investigators have failed to detect glutamate synthase in variety of eukaryotes including fungi (Branchley et al., 1973; Desai and Modi, 1977; Tempest et al., 1973). NAD⁺-alanine dehydrogenase has been reported to be absent in Claviceps purpurea (Fr.) Tul. (Rehacek et al., 1977).

The fall in intracellular level of ammonia during the intensive alkaloid production and rise in glutamine synthatase activity can be correlated (Fig.IV.4). Ammonia has been found to be one of the substrate for glutamine synthatase. Glutamine synthatase is an enzyme of central importance in nitrogen metabolism because of its function to produce glutamine, an essential amino acid. This enzyme has been known to be involved in regulation of macromolecules in bacteria (Taylor et al., 1974) and fungi (Dunn-coleman and Garrett, 1980). Glutamine has been
reported as amino donor for the synthesis of other compounds including tryptophan (Rehacek et al., 1977). The level of tryptophan (Fig. IV.4) in the cell during shake cultivation of Claviceps sp. SD-58 shows parallel dynamics to the level at glutamine synthetase. Glutamine, a product of glutamine synthetase has generally been documented as the amino group donor in the synthesis of anthralinate (Zalkin and Murphy, 1975) by following reaction:

$$\text{Chorismate} + \text{NH}_4 \rightarrow \text{Anthralinate} + \text{Pyruvate}$$

Tryptophan has been established as an ergoline ring precursor (Mothes et al., 1958; Groger et al., 1959; 1960; Taber and Vinning, 1958; Arcamone et al., 1962; Floss and Mothes, 1964; Rao and Patel, 1974; Krupinski et al., 1976)

Thus, the results obtained lead us to believe that asparagine plays an important role in manifestation of Claviceps species, Strain SD-58 genotype. The data obtained suggests that the metabolism of asparagine via asparaginase results into the accumulation of ammonia in the early phase of fermentation. During the intensive alkaloid producing phase this ammonia is being metabolized through glutamine synthetase causing higher tryptophan synthesis.
The accumulation of tryptophan interum may be one of the factors for over production of alkaloid during the submerged cultivation of *Claviceps* species, Strain SD-58 in asparagine containing medium.
REFERENCES


