CHAPTER FOUR

MICROBIAL HYDROLYSIS OF CRUDE GLYCOALKALOID OF *S. KHASIANUM* CLARKE BY ASPERGILLUS NIGER.

INTRODUCTION

Sometimes micro-organisms can be utilized for biological conversion of various substrate for the preparation of Pharmacologically active constituents (Holland and Gregg, 1979; Kurdosova et al. 1975; Perepelista and Mintya, 1978; Trease and Evans, 1978). As micro-organisms are easy to propagate in the laboratory, the knowledge of biological conversion sometimes may be utilized during commercial production of pharmaceutically active compounds.

Solasodine is produced after acid hydrolysis of its various glycosides but an appreciable amount of solasodine is lost due to its conversion to undesirable solasodiene, a dehydrated product of solasodine (Briggs et al. 1942; Labenskii and Koretskaya, 1961; Maiti et al. 1965; Weston, 1976).

Various improved chemical methods have been suggested by different workers to minimize the quantity of undesirable solasodiene during acid hydrolysis of glycoalkaloids (Cioga et al. 1967; Segal et al. 1978; Weston, 1976; Choudhuri et al. 1979).

It has been observed that during microbial hydrolysis of crude glycoalkaloid obtained from berries of <u>S. khasianum</u> Clarke caused by <u>Aspergillus niger</u> no 'diene' was produced.

In the present investigation, attempts have been made to determine the chemical identification of the product obtained during microbial hydrolysis of the glycoalkaloid of S. khasianum Clarke with the help of a strain of A. niger and to determine the optimum condition of engyme activity on glycoalkaloid for the production of solasodine.

MATERIALS AND METHODS

Materials:

Crude glycoalkaloid was isolated from berries of S. khasianum Clarke following the procedure of flow sheet table 4. A. niger was collected from IARI, New Delhi and was maintained on malt Agar media.

Methods:

Adjustment of pH of alkaloid media: Glycoalkaloid (2 gms) was dissolved in saturated solution of NaCl and the alkaloid solution was added to malt agar media. pH of the medium was maintained at 5.8 using 0.1 N HCl and 0.1 N NaOH solutions. Fungus mycelium and spores were inoculated and allowed to grow in an incubator at $36^{\circ} \pm 1^{\circ}C$.

Isolation and purification of steroidal alkaloid obtained from the fungal culture:-

The five days old culture media was treated

throughly with 2% HCl and filtered. The filtrate was basified with ammonia and centrifuged. The semi-solid mass was taken in MeOH and concentrated under reduced pressure. The concentrated mass was column chromatographed over alumina with non-polar to polar solvents according to the procedure mentioned earlier (Chapter two).

Preparation of the stock solution of glycoalkaloid:

Glycoalkaloid was dissolved saturated aqueous solution of NaCl to prepare the stock solution (1000 ppm).

For extraction of enzymatic factor from the culture media, Phosphate buffer was utilized.

Preparation of Buffer solution: Potassium phosphate buffer was prepared after adding 3.5 ml of 0.2 gm/1L potassium hydroxide to 50 ml. of 0.2 gm/1L potassium hydroxide phosphate and diluted to 100 ml. for constant 5.8 pH.

Extraction of Enzyme: Fungal culture along with the organism was taken in a chilled motorpestle containing chilled buffer solution and macerated throughly. Brown concentrated mass was then centrifuged in cold and the supernatant was decented off through fine cloth.

Enzyme activity and estimation of steroidal alkaloid:

5 ml. of glycoalkaloid solution was taken in a number of conical flask and 2.5 ml. of enzyme extract

was added to each of the conical flask and kept in incubator at $36^{\circ} \pm 1^{\circ}C$. Every after ten minutes a flask was taken out and the activity of enzyme was stopped adding 2 ml. of hot methanol. It was evaporated to dryness under reduced pressure and dissolved in 5 ml. of 2% HCl and filtered. The filtrated was basified with ammonia at pH 10 and was centrifused. The semi-solid mass was dissolved in hot MeOH and subjected to TLC on silica gel (Kisel gel G) and developed in the solvent system [Benzene: Chloroform (40:60 v/v)] along with the authentic sample of solasodine and glycoalkaloid. The developed chromatogram having solasodine (Rf. 0.27) and glycoalkaloid (Rf. 0.00), was air dried and the silica gel was scraped off from the localized zone corresponding to the authentic solasodine and glycoalkaloid being detected under UV light. The scrapped silica gel was taken separately in test tube containing 5 ml. of methanol and boiled for 2-3 min. Methanolic extract was centrifuged. The filtrate was concentrated and the volume was made upto 5 ml. with methanol. 1 ml. of each filtrate was treated with 1 ml. of resorcinol and 0.5 ml. of Conc. H_2SO_4. OD value was measured in a spectrocolorimeter at 513 nm corresponding to the absorption maxima for solasodine. The percentage of solasodine was calculated following the standard curve and the unhydrolysed glycoalkaloid was estimated colorimetrically at the same weave taking solasodine as the standard.

RESULTS AND DISCUSSIONS.

Residue (430 mg) obtained after column chromatography over alumina represented in the table 16.

Table 16. Residue obtained from methanolic extract of fungal culture (*Aspergillus niger*) after microbial hydrolysis of crude glycoalkaloid.

Eluent	Sub-fractions	Residue on evaporation.
Petroleum ether (60°-80°C)	1 - 10	Oily (trace) with deep brown pigment.
-do-	11 - 15	-do- (trace)
-do-	16 - 20	nil
Petroleum ether: (60°-80°C) Benzene		
3 : 1	21 - 25	Brown pigment.
1 : 1	26 - 30	-do- (trace)
1 : 3	31 - 35	nil

Table 16 (Contd.)

Eluent	Sub-fractions	Residue on evaporation.
Benzene : Chloroform		
3 : 1	36 - 40	Crystal (trace)
1 : 1	41 - 45	Crystal (trace)
1 : 3	46 - 50	Crystals
Chloroform	51 - 55	-do-
-do-	56 - 60	-do-
-do-	61 - 65	-do- (trace)
-do-	66 - 70	nil
Chloroform : Methanol		
3 : 1	71 - 75	Crystal (trace)
1 : 1	76 - 80	Crystal
1 : 3	81 - 85	-do-
-do-	86 - 90	-do- (trace)
Methanol	91 - 95	Crystals (trace)
-do-	96 - 100	-do-
-do-	101 - 105	nil

Sub-fractions 36 - 65

It was recrystallized from acetone when colourless hexagonal plates of crystals were found. A small fraction of the crystals was dissolved in hot methanol and

subjected to TLC on silica gel (Kisel gel G.E.Merck) and was found to be identical in behaviour with the authentic solasodine and this has been represented in the following table 17.

Table 17. Chromatographic behaviour of Solasodine obtained after microbial hydrolysis of glycoalkaloids by <u>Aspergillus</u> <u>niger.</u>

Compound identified.	Rf. value in different solvent systems.			Colour with	
	Chloroform: Acetone (99:1,v/v)	Butanol: Acetic Acid: water (10:3:8, v/v).	Chloroform: Methanol (19:1,v/v)	Vanillin H_2SO_4	$SbCl_3$ in chloroform.
Substance obtained after biological hydrolysis.	0.28	0.91	0.24	Green to pink bluish	Intense crimson
Authentic solasodine.	0.28	0.91	0.24	Green to pink bluish.	Intense crimson.

A developed chromatogram was sprayed with O-phosphoric acid (1 volume of O-phosphoric acid was diluted

with 1 volume of water) and the plate was heated at 120°C for 10 minutes. The bluish spot of the steroidal alkaloid (Solasodine) were intensified when the chromatogram was again sprayed with freshly prepared phosphomolybdic acid (1.5 g. phosphomolybdic acid in 100 ml. ethanol) and heated at 120°C for 5 minutes. Authentic sample of solasodine and the isolated crystals produced brown fluorescence on a developed chromatograms under UV light and both of them showed violet colouration with resorcinol acetic acid and Conc. H_2SO_4.

Isolated crystals and authentic sample gave amber red colour with Conc. H_2SO_4 in cold. Authentic solasodine and isolated crystals dissolved in hot alcohol gave characteristic intense greenish yellow fluorescence similar to that of heavy lubricating oil when they were treated with Conc. H_2SO_4. Characterization of the isolated chemical was finally confirmed with m.p. (199.5 - 201°C), mmp (undepressed) and superimposable infra red spectra (in nujol) at 3420, 3320, 3240, 1670, 1460, 1370, 1350, 1290, 1250, 1190, 1140, 1070, 1050, 1020, 1000, 970, 960, 910, 980, 880, 840, 790, and 730 cm^{-1} (Fig.22) with the authentic marker of solasodine obtained from Indian Institute of Experimental Medicine, Calcutta.

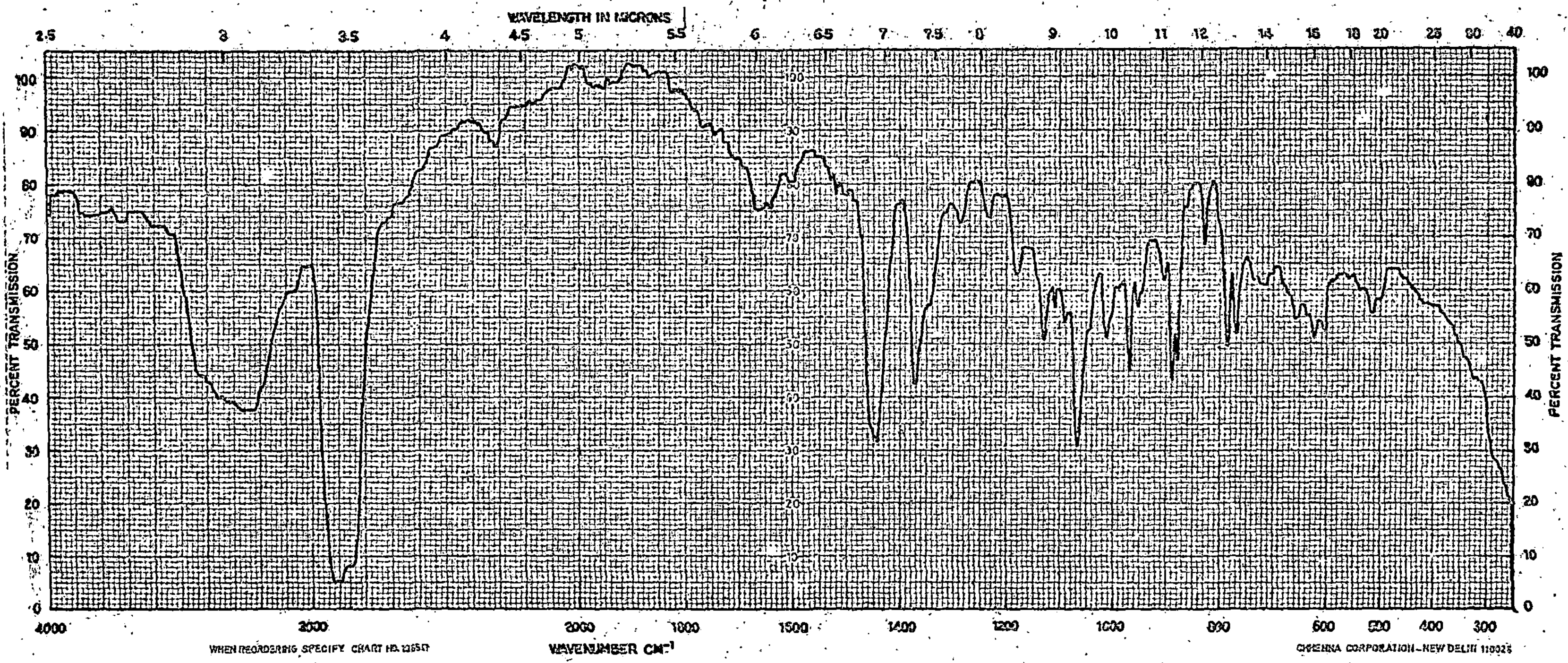

Fig. 22 Infrared spectrum of solasodine obtained during the microbial hydrolysis of glycoalkaloid of *S. khasianum* Clarke

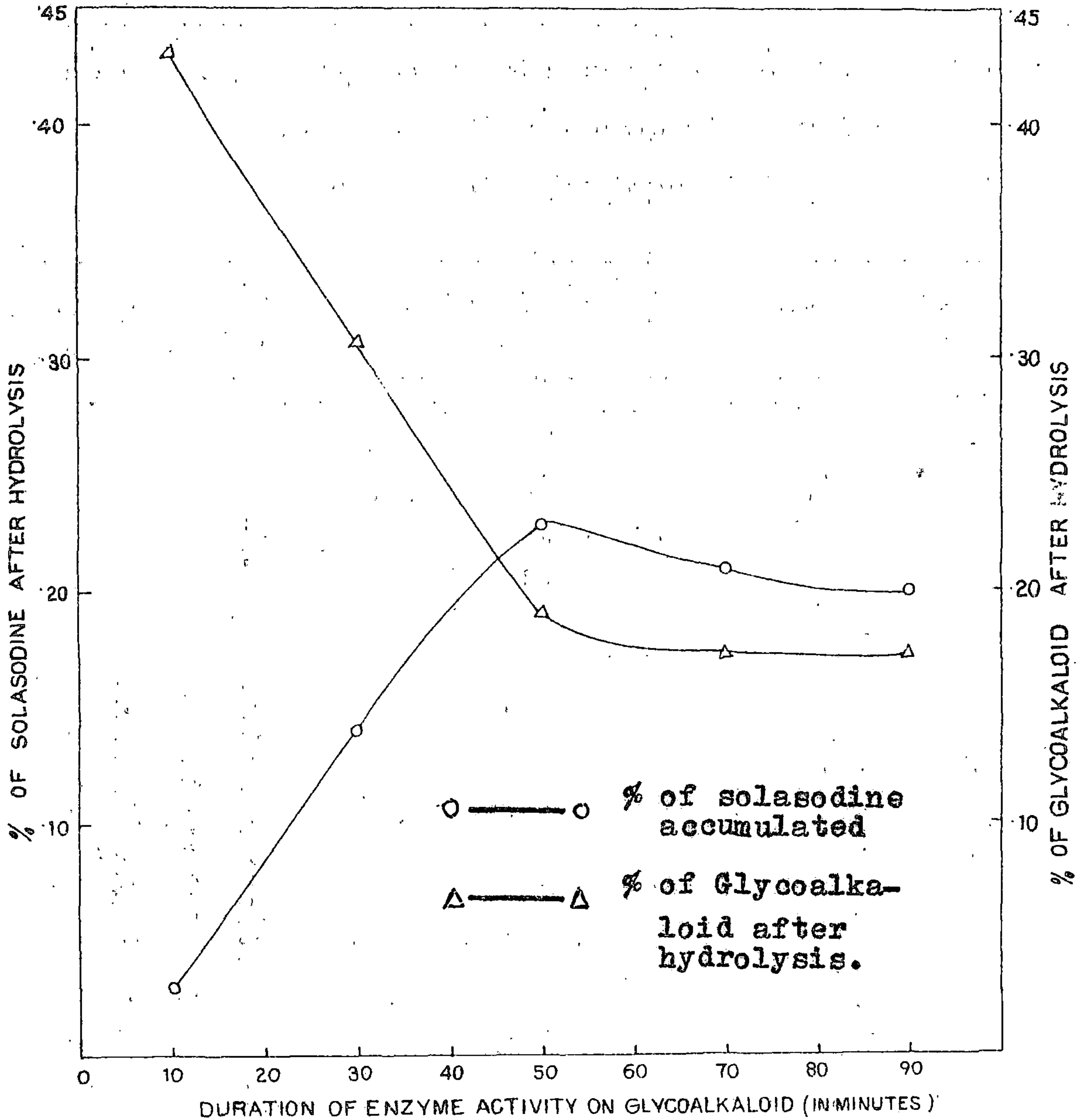

Fig. 23 Effect of crude enzyme isolated from the culture of *Aspergillus niger* on the hydrolysis of crude glycoalkaloid of *S. khasianum* Clarke.

Sub-fractions 71-100

The sub-fractions of the eluent were collected and evaporated to small volume. Pure crystals were obtained from chloroform-methanol mixture and that was recrystallized from acetone. A small fraction of this compound was dissolved in hot methanol and subjected to TLC on silica gel G (kisel gel G.E.Merck) and developed in a solvent system n-Butanol:Acetic acid:water (10:3:8, v/v). The developed chromatogram was treated in iodine vapour and found to be identical behaviour with the authentic sample of solasonine (Rf. 0.75) and solamargine (Rf. 0.80). Besides solasonine and solamargine, the developed chromatogram showed two more spots, the Rf. values of which were 0.63 and 0.68 respectively. Solasonine and solamargine were further characterized after comparing m.p., mmp (undepressed), TLC behaviour and IR spectrum with the authentic samples.

From this experiment, it can be concluded that the steroidal glycoalkaloid of S. khasianum Clarke was enzymatically hydrolysed to solasodine by Aspergillus niger without any formation of solasodiene. The production of solasodine during the microbial hydrolysis of glycoalkaloid is very much similar to that obtained during the acid hydrolysis of glycoalkaloid as observed by several workers (Briggs et al. 1942; Labenskii and Koretskaya, 1961;

Maiti et al. 1965; Weston, 1976; Segal et al. 1978; Choudhuri et al. 1979) excepting that the 'diene form' was not detected during this biological hydrolysis, though the conversion of 30 - 40% of solasodine to solasodiene was observed (Maiti et al. 1968) in acid hydrolysis of glycoalkaloid. A successful attempt of microbial hydrolysis of glycoalkaloid of S. elaegnifolium Cav. by Aspergillus niger has also been made by Rodriguez et al. (1979).

The activity of the enzyme, extracted from the fungal culture, on the hydrolysis of glycoalkaloid to yield free solasodine was noted to increase gradually to yield maximum amount (0.23%) of solasodine at 50 minutes during the investigation of enzyme on glycoalkaloid (Fig. 23). The yield of solasodine was noted to decreased gradually (0.20%) during 90 minutes of the treatment.

This observation supports the experimentation of Rodriguez et al. (1979) who demonstrated that the solasodine, obtained from microbial hydrolysis of crude glycoalkaloid of S. elaegnifolium Cav. was gradually decreased in the culture media. He suggested that the solasodine was converted to 16-dehydropregnenolone acetate after its (solasodine) formation through microbial

hydrolysis of glycoalkaloid. This type of conversion and other factors which are responsible for production of maximum at amount of solasodine along with the characterization of enzyme wheather extracellular or intracellular in nature, are yet to be worked out in case of <u>S. khasianum</u> Clarke. As the enzymatic factor removes the sugar moiety from the glycoalkaloid, the enzyme is supposed to be glycosidase in nature. As similar glycosidase has been noted to occur in the sprout of <u>S. taberosum</u> L. (Petrotschenko, 1957), the enzyme is expected to be distributed from higher plants to at least lower organism like <u>Aspergillus niger.</u>

SUMMARY.

With the help of Aspergillus niger, microbial hydrolysis of crude glycoalkaloid obtained from the berries of S. khasianum Clarke was performed. During the microbial hydrolysis only solasodine was detected. Identification of the compound was done after comparing the m.p., mmp (undepressed), TLC behaviour and IR spectrum with the authentic solasodine.

Enzyme activity of the fungus on the glycoalkaloid was studied. It was noted that the enzyme factor was soluble in phosphate buffer at 5.8. The maximum production of solasodine was observed during the enzyme activity for 50 minutes at $36^{o} \pm 1^{o}C$.

The enzyme was noted to glycosidase in nature.