LEVELS OF PROTEIN AND ESSENTIAL AMINO ACIDS IN *RHIZOCTONIA SOLANI* KUHN GROWN ON WOOD PULP

by D. S. CHAHAL *and* G. D. MUNSHI, *Department of Microbiology, Punjab Agricultural University, Ludhiana (Punjab)*

and

A. K. CHOPRA, *Department of Chemistry and Biochemistry, Punjab Agricultural University, Ludhiana (Punjab)*

(*Received 26 March 1974, after revision 20 January 1975*)

Utilization of cellulosic material, wood pulp, was exploited for the production of fungal protein by *Rhizoctonia solani*. The fungal protein produced, indicated the presence of all the esseential amino acids in good amount. Maximum production of protein and amino acids was obtained with urea as nitrogen source at the concentration of 600 mg N/l by incubating cultures for 96 hr.

INTRODUCTION

Chahal and Gray (1968) explored the possibility to use cellulosic material, wood pulp, for its conversion into fungal protein to alleviate the protein shortage in the world. They have reported that out of 44 different cellulolytic fungi only four, *viz*, *Myrothecium verrucaria, Chaetomium globosum, Rhizoctonia solani* and *Trichoderma* sp. were able to grow well on wood pulp and produced maximum amount of fungal protein. Chahal *et al.* (1970) further reported the presence of all the essential amino acids in these four cellulolytic fungi with the only exception of threonine which was absent in *Trichoderma* sp. *R. solani* was better in respect of total protein and amino acids contents than the other three fungi. Chahal *et al.* (1969) indicated that *R. solani* was able to grow better on wood pulp than on any other cellulosic material and produced maximum amount of fungal protein. The present study was undertaken to find the best conditions for synthesis of protein and essential amino acids by *R. solani* when grown on wood pulp.

MATERIALS AND METHODS

The Basal synthetic medium used was same as described by Chahal and Gray (1968). The composition of the medium is as follows :

Nitrogen source[1]	400 mg N/l	Vitamin solution[2]	1 ml/l
KH_2PO_4	2.5 g/l	Trace elements solution[3]	1 ml/l
$MgSO_4.7H_2O$	1.25 g/l	$ZnSO_4$ solution (44 g/l)	1 ml/l
Dextrose	1.00 g/l	$FeCl_3$ solution (1.92 g/l)	1 ml/l

1. The source and amount of nitrogen used in different experiments has been indicated at their respective places.

2. *Composition of vitamin solution per litre* : Thiamine hydrochloride, 100 mg; Pyridoxine hydrochloride, 50 mg; Calcium pentothenate, 200 mg; P-aminobenzoic acid, 50 mg; Nicotinamide, 200 mg; Inositol, 400 mg; Riboflavin, 50 mg.

3. *Composition of trace elements solution per litre* : boric acid, (H_3BO_3) (A.R.), 114 mg; ammonium molybdate (A. R.), [$(NH_4)_6$ $Mo_7O_{24}4H_2O$,] 480 mg; cupric sulphate (A.R.) ($CuSO_4.5H_2O$), 780 mg; manganese chloride (A.R.) ($MnCl_2.4H_2O$), 114, mg.

Wood pulp was supplied by M/s Shree Gopal Mills, Ltd., Yamunanagar, Ambala (Haryana) which was prepared by sulphate process from wood of mixed conifers. This wood pulp was mainly α-cellulose almost free from lignin. The *p*H of the medium was so adjusted that it was 4.5 after the addition of wood pulp and autoclaving. The quantity of different nitrogen salts was used to give equivalent amount of 400 mg N/1. When urea was used as N source, the medium was autoclaved separately and urea being added later from stock solution (20 mg N/ml). The stock solution was sterilized by filtering through bacteria-proof Seitz filter. The required volume of filter sterilized solution was added aseptically to give desired amount of N.

Powdered wood pulp (60 mesh) weighing 750 mg was added into each Erlenmeyer flask of 250 ml capacity containing fifty ml of medium, plugged with non-absorbant cotton wool, and autoclaved at 15 psi for 20 min.

The inoculum was prepared by blending the mycelium in the Waring blender. Stale medium was removed by decanting and the mycelium was washed twice with sterile distilled water to remove the traces of medium and metabolites. The uniform mycelial suspension was prepared aseptically in 100 ml of sterile distilled water.

Two ml mycelial suspension was used for inoculating each flask. The inoculated flasks were kept on reciprocating shaker with a stroke of 2.5 cm and 100 strokes per min. The flasks were incubated for 4 days at room temperature of 25 to 28°C.

The growth was measured as dry weight of fungal mycelium including undigested wood pulp and it was further estimated in terms of crude protein synthesized. The crude protein in fungal mass was calculated by determining the total nitrogen by microkjeldahl method. The figures thus obtained were multiplied by the factor 6.25.

Twenty ml of dried fungal mycelium was hydrolyzed with 6N HCL overnight in a sealed tube at 110°C. The excess acid was removed by drying it under vacuum and volume made to 5 ml.

The qualitative analysis of amino acids was done by thin layer chromatography by the method as outlined by Brenner and Niederwiesser (1960). Total number of ninhydrin positive spots developed on chromatoplate was recorded. The amino acids in the sample were identified by comparing their R_f values with those of amino acids in known mixture.

For quantitative estimation of separated amino acids, the colorimeteric procedure described by Voigt *et al.* (1965) and Esser (1965) with slight modification was followed. After the colour development the chromatoplates were brought to room temperature, the ninhydrin positive spots corresponding to the R_f values of the known essential amino acids were scrapped with micro-spatula and transferred to curcible. Five ml of methanolic solution of cadmium acetate and ninhydrin was used for dissolving the scrapped fractions. The clear solution was obtained by removing silica gel by centrifuging at 3,000 rmp for 5 min. Optical density was measured with Spectronic 20 spectrophotometer at the 5000Å. The quantity of each amino acid present was determined by plotting the readings so obtained on the standard curve of respective amino acids.

Standard curves of amino acids were prepared by developing the chromatoplates with concentrations ranging from 2.5 to 25 μg of individual amino acids. The spots were taken in methanolic solution of cadmium acetate and ninhydrin in

the same way as described above. The optical density was plotted against concentration to draw the standard curve.

Tryptophan was estimated in alkaline hydrolysate by the method of Smith and Agiza (1951).

Experimental Results

Effect of different nitrogen sources

Remarkable differences were recorded in the crude protein percentage and total protein production with the utilization of different forms of nitrogen (Table I).

The highest percentage of crude protein and total protein production indicated the preference for urea as nitrogen source followed by potassium nitrate while in case of ammonium chloride protein percentage was very low. The high amount of dry matter obtained in case of ammonium chloride and low percentage of protein indicated that lot of wood pulp was not converted into mycelial mass.

The quantities of all the essential amino acids were highest in case of urea, followed by potassium nitrate and ammonium nitrate. All the amino acids differed quantitively with different nitrogen source except histidine. The quantity of which remained almost the same in all the nitrogen sources studied.

Effect of different levels of nitrogen

The utilization of carbon and synthesis of fungal material is largely dependant on the carbon-nitrogen ratio. Thus to find out the optimum nitrogen dose for maximum production of protein and amino acids, the different nitrogen levels (200 to 1000 mg N/l) were studied. Urea was used as nitrogen source as it gave the best results (Table I). The data presented in Table II clearly indicate that 600 mg N/l surpassed all other levels with respect to protein percentage and total protein production closely followed by 800 mg N/l. Total protein as well as crude protein percentage was lowest at the level of 200 mg N/l. The higher concentration of nitrogen (1000 mg N/l) adversely affected protein percentage and total protein production.

Total number of ninhydrin positive spots detected were 19 in 200 mg N/l and the number increased to 23 with the increase in nitrogen concentration from 400 to 800 mg N/l. Further increase in the concentration of nitrogen adversely affected the number of ninhydrin positive spots. Almost all the essential amino acids increased with the increase in nitrogen concentration upto 600 mg N/l but the amount of arginine and histidine continued to increase upto 800 mg N/l. Further increase in the nitrogen concentration decreased the amount of all the essential amino acids except histidine. Methionine was not detected at the highest nitrogen concentration. As urea at the level of 600 mg N/l gave the best results thus this concentration was selected for further investigations.

Effect of different periods of incubation

Pillai and Srinivasan (1956) and Falina *et al.* (1963) observed quantitative difference in the level of individual amino acids depending upon the age of cultures.

TABLE 1

Effect of different nitrogen sources[1]

Nitrogen source	Final *p*H	Wt. of mycelium & undigested wood pulp (mg)	Crude protein (%)	Total protein per flask (mg)	Total No. of ninhydrin positive spots	Arginine	Histidine	Phenylalanine	Methionine	Leucine and/or isoleucine	Valine	Lysine	Threonine	Tryptophan
Urea	5.4	427	19.3	82	24	2875[2]	1200	400	1000	1775	1500	575	1000	376
						(14.7)[3]	(6.1)	(2.0)	(5.1)	(9.1)	(7.7)	(3.0)	(5.1)	(1.9)
Potassium nitrate	7.1	401	18.4	74	20	2500	1200	300	1000	1500	1200	475	800	368
						(13.5)	(6.5)	(1.6)	(5.4)	(8.1)	(6.5)	(2.6)	(4.3)	(2.0)
Ammonium nitrate	5.1	440	16.6	73	25	2500	1000	200	825	1375	1200	275	600	368
						(15.1)	(6.0)	(1.2)	(5.0)	(9.3)	(7.2)	(1.7)	(3.6)	(2.2)
Ammonium chloride	3.8	729	7.0	51	17	850	1000	100	675	550	450	100	200	200
						(12.2)	(14.3)	(1.4)	(9.7)	(7.8)	(6.4)	(1.4)	(2.8)	(2.9)

1, 400 mg N/l; 2, mg/100 g mycelium (plain figures); 3, g/16 g nitrogen (figures in parentheses).

TABLE II

Effect of different levels of nitrogen[1]

Level of nitrogen	Final *p*H	Wt. of mycelium & undigested wood pulp (mg)	Crude protein (%)	Total protein per flask (mg)	Total No. of ninhydrin positive spots	Arginine	Histidine	Phenylalanine	Methionine	leucine and/or isoleucine	Valine	Lysine	Threonine	Tryptophan
200 mg N/l	5.4	538	10.4	56	19	1250[2]	400	300	675	1100	750	200	400	340
						(11.9)[3]	(3.8)	(2.9)	(6.5)	(10.5)	(7.1)	(1.9)	(3.8)	(3.2)
400 mg N/l	5.6	461	20.8	96	23	2500	1400	400	1137	1912	1500	575	1000	376
						(12.0)	(6.7)	(1.9)	(5.5)	(9.2)	(7.2)	(2.8)	(4.8)	(1.8)
600 mg N/l	6.4	435	24.4	107	23	3300	1800	500	1000	2175	1500	750	1200	372
						(13.5)	(7.4)	(2.0)	(4.1)	(9.0)	(6.2)	(3.6)	(4.9)	(1.5)
800 mg N/l	6.6	435	23.6	103	23	3725	2000	500	825	1912	1200	650	800	368
						(15.7)	(8.4)	(2.1)	(3.5)	(8.0)	(5.0)	(2.7)	(3.4)	(1.6)
1000 mg N/l	6.6	429	22.1	95	21	3300	2000	400	—	1775	1200	575	800	352
						(14.9)	(9.0)	(1.8)		(8.0)	(5.4)	(2.6)	(3.6)	(1.6)

1, Urea used as Nitrogen source; 2, mg/100 g mycelium (plain figures); 3, g/16 g nitrogen (figures in parentheses).

TABLE III

Effect of different periods of incubation

Incubation (hr)	Final *p*H	Wt. of mycelium & undigested wood pulp (mg)	Crude protein (%)	Total protein per flask (mg)	Total No. of ninhydrin positive spots	Arginine	Histidine	Phenylalanine	Methionine	Leucine and/or isoleucine	Valine	Lysine	Threonine	Tryptophan
48	6.2	505	19.7	99	18	2075[1]	800	400	—	825	875	575	600	320
						(10.6)[2]	(4.1)	(2.0)		(4.2)	(4.5)	(3.0)	(3.0)	(1.6)
72	6.3	477	22.7	108	24	3300	1000	700	825	1925	1200	575	600	342
						(14.5)	(4.4)	(3.1)	(3.7)	(8.5)	(5.3)	(2.6)	(2.6)	(1.5)
96	6.3	439	24.1	106	24	3725	1600	600	1000	2050	1350	750	1000	368
						(15.7)	(6.7)	(2.5)	(4.2)	(8.6)	(5.7)	(3.2)	(4.2)	(1.6)
120	6.4	398	23.2	92	24	3725	1375	600	1000	1925	1350	750	800	360
						(16.0)	(5.9)	(2.6)	(4.3)	(8.3)	(5.8)	(3.2)	(3.4)	(1.5)
144	6.7	380	20.1	76	24	3300	1375	200	850	1350	900	575	800	340
						(16.5)	(6.9)	(1.0)	(4.3)	(6.8)	(4.5)	(2.9)	(4.0)	(1.7)

1, mg/100 g of mycelium (plain figures); 2, g/16 g nitrogen (figures in parentheses); 3, 600 mg N/l in the form of urea.

Therefore, it was decided to find out optimum incubation period for maximum protein and amino acid production in *R. solani.*

Continuous fall in dry weight of mycelium and undigested wood pulp with the increase in incubation period indicated the increase in utilization of wood pulp (Table III). Percentage of protein was highest after 96 hr while total protein reached the highest level even after 72 hr of incubation.

Total number of ninhydrin positive spots recorded after 48 hr of incubation were 18 but in all other cases it was 24. All the essential amino acids continued to increase upto 96 hr of incubation thereafter the amount slightly decreased. Methionine could not be detected in early period of growth i.e. upto 48 hr.

Discussion

Urea was the best source of nitrogen for maximum protein and amino acid production. Amongst inorganic forms of nitrogen nitrate N was preferred over ammonium N. Sinden *et al.* (1948) have reported that the rate of breakdown of cellulose by *Myrothecium verrucaria* with a ratio of one ammonium to 24 nitrate ions was almost three times than that of when N was present completely in ammonium form.

Utilization of nitrogen and total protein and amino acids production increased with the increase in the nitrogen (urea) concentration to a certain level but with further increase in concentration their contents decreased.

Inhibitory effect of high doses of urea might be attributed to the accumulation of NH_4^+ released by the breakdown of urea. Toxic and inhibitory effect of high doses of ammonium salts or NH_4^+ on fungi had been reported by many workers (Fries 1949; Keitt and Palmiter 1937; McCallan and Weeden 1940; Neal 1936; Neel *et al.* 1932, 1933; and Pratt 1924).

Total protein production and quantities of amino acids increased with the increase in incubation period upto 96 hr, thereafter, decrease in protein and amino acids production was noticed. This might be due to autolyzation of mycelium. Smithies (1953) had reported that autolysing mycelium of *Penicillium griseofulvum* undergoes an extensive breakdown of chitin, carbohydrates and protein.

References

Brenner, M., and Niederwiesser, A. (1960). Chromatography of amino acids on thin layers adsorbants. *Experentia,* **16,** 378–387.

Chahal, D. S., and Gray, W. D. (1968). Growth of selected cellulolytic fungi on wood pulp, pp. 584–593. *In :* Biodeterioration of Materials-Microbial and Allied Aspects (Eds. A. H. Walter and J. S. Elphic). Elsevier Publishing Co. Ltd., England.

Chahal, D. S., Gray, W. D., and Munshi, G. D. (1970). Amino acid composition of cellulolytic fungi grown on wood pulp. *Indian J. Microbiol.*, **10,** 23–26.

Chahal, D. S., Munshi, G. D., and Cheema, S. P. S. (1969). Fungal protein production by *Rhizoctonia solani* when grown on cellulose as sole source of organic carbon. *Proc. natn. Acad. Sci. India.*, **B39,** 287–291.

Esser, K. (1965). Thin layer chromatography for quantitative determination of amino acids and amino sugars. *J. Chromatog.*, **18,** 414–416.

Falina, N. N., Naslove, R. A., and Yakimov, P. A. (1963). On the amino acid composition of the mycelium of fungus *Hydnum septentrionate*. *Mikrobiologiva*, **32**, 223–226.

FOSTER, J. W. (1949). Chemical Activities of Fungi. Academic Press, New York. pp. 439.

Fries, N. (1949). Culture studies in genus *Mycena*. *Syenak Botan. Tidskr.*, **43**, 316–342.

Keitt, G. W., and Palmiter, D. H. (1937). Gradient fungicides in relation to apple scab control. *Phytopathology*, **27**, 133.

McCallan, S. E. A., and Weedon, F. R. (1940). Toxicity of amonia, chlorine, hydrogen cyanide, hydrogen sulphide gases, II. Fungi and Bacteria. *Contrib. Boyce Thompson Inst.*, **11**, 331–342.

Neal, D. C. (1936). Concentration of ammonia necessary in low lime phase of Houston clay soil to kill the cotton root-rot fungus, *Phymatotrichum omvivorum*. *Phytopathology*, **26**, 1031–1032.

Neal, D. C., Webster, R. E., and Gunn, K. C. (1932). Treatment of cotton root-rot with ammonia. *Science*, **75**, 139–140.

Neal, D. C., Webster, R. E., and Gunn, K. C. (1933). Growth of cotton root-rot fungus in synthetic media and toxic effect of ammonia on the fungus. *J. Agri. Res.*, **47**, 107–118.

Pillai, N. C., and Srinivasan, K. S. (1956). Amino acid metabolism of *Aspergillus flavus*. *J. Gen. Microbiol.*, **14**, 248–255.

Pratt, C. A. (1924). Staling of fungal cultures. II. The alkaline metabolic products and their effect on growth of fungal spores. *Ann. Botany*. **38**, 615.

Sinden, J. W., Mix, J., and Siu, R. G. H. (1948). Microbiological Series Report No. 8 Philadelphia, U. S. Army Quartermaster.

Smith, A. M., and Agiza, D. H. (1951). The amino acids of several grassland species, cereals and bracken. *J. Sci. Fd. Agric.*, **2**, 503–520.

Smithies, W. R. (1953). The lysing action of enzymes on a sample of mycelium of *Penicillium griseofulvum*. *Biochem. J.*, **55**, 346–350.

Voigt, S., Solle, M., and Koitzer, K. (1965). Thin layer chromatographic separation of aminobutyric acids from the brain extracts. *J. Chromatog.*, **17**, 180–182.