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USE OF DEPROTEINISED LEAF JUICE OF *MEDICAGO SATIVA* L. FOR THE **PRODUCTION OF α**-AMYLASE

S Iliyas*

Department of Botany
Poona College of Arts, Science and Commerce, Camp. Pune- MS 41100, India

Abstract

Leaf protein concentration (LPC) is a good source of cyanocobalamine (B_{12}), ascorbic acid (vitamin C) and folic acid (vitamin B_9). After isolation of LPC from leaves the remaining by product is deproteinised leaf juice (DPJ) which is rich in water soluble carbohydrates, free amino acids, minerals, lipids and vitamins. The dry matter (DM) and nutrient composition of DPJ varies from species to species. The DM content in this fraction was found between 1.2 to 4.0%. Six fungi were grown on the DPJ expressed from *Medicago sativa, Anethum graveolens, Spinacia oleracea, Trigonella foenium-graecum, Coriandrum sativum* and also on conventional GN medium to evaluate the suitability of DPJ as a medium for fungal growth and subsequent production of ∝-amylase. The efficiency of DPJ as a medium for fungal growth was evaluated and its value in microbial biotechnology for the production of ∝-amylase was tested. Increase in concentrate of flour in the DPJ there was simultaneous increase in the mycelial dry weight (MDW) production of the six fungi under investigation. On DPJ alone, the yield of MDW was between 76.0 and 89.5 mg which increased to 164.5, 131.0 and 117.0 mg respectively under the influence of the flours of wheat, sorghum and maize respectively. Maximum response to the enrichment of DPJ by flour was noticed with *A. niger.* As with the increase in growth of fungi with increasing concentration of flour, the activity of amylase also increased. On an average it was 34 - 96 U/ml when the fungi were grown on DPJ alone, which increased to 301, 265 and 276 U/ml under the influence of flours.

Key words: Amylase, DPJ, LPC, Lucerne, *Aspergillus*

Introduction

The deproteinised leaf juice is rich in water soluble nutrients present in the leaves. It contributes to more than 50% of the fresh weight from green foliage which is fractionated for the production of pressed crop residue (PCR) and leaf protein concentration (Iliyas 2011). It is rich in water soluble carbohydrates, free amino acids, minerals, lipids and vitamins (Iliyas and Badar 2010a). It also contains small fraction of protein (Iliyas and Badar 2010). The effect of additives on chlorophyll content in wet LPC prepared from juice of *Medicago sativa* L. is more effective (Iliyas 2010, Iliyas and Mungikar 2003). Some bioinformatical aspects protein isolated from *Medicago sativa* L. *viz*. A comparative study of protein structure visualization tools for various display capabilities were studied (Ansari and Iliyas 2011). A comparative study of different properties provided by protein structure visualization tools (Iliyas and Ansari 2013). Implementation of image processing in agriculture sector. Production of amylase of DPJ of four different plants (Iliyas 2013).

This product, with 4 to 5% solids, contains large proportion of nitrogen and phosphorus. The dry matter and nutrient composition of DPJ varies from species to species. At Rothemsted Experimental Station in UK the DM content in this fraction was found to be between 1.2 to 4.0%. On an average, the N and carbohydrate content in DM of DPJ are 3% and 40% respectively. The dominant monosaccharides in DPJ are glucose and

*Author for correspondence: sayyed_iliyas@yahoo.com

fructose. However, the contents of these reducing sugars in DPJ are subject to a great change, depending upon the species used for Green Crop Fractionation (GCF) and maturity of the plants used. This by-product should be disposed properly in order to avoid local environmental bio-pollution (Iliyas and Mungikar 2005). Proper exploit of the DPJ is also useful in making DPJ as a commercial by product (Josephin and Iliyas 2005, Iliyas 2014a, Iliyas 2014b). During present investigation the efficiency of DPJ as a medium for fungal growth was evaluated and its value in microbial biotechnology for the production of enzyme ∝-amylase was tested.

Materials and Methods

Preparation of DPJ

The green foliages from *Medicago sativa, Anethum graveolens, Trigonella foenium-graecum, Coriandrom sativum* and *Spinacia oleracea* were used for fractionation. The juice released during fractionation was employed for the preparation of LPC by heat coagulation and the DPJ released after precipitation and isolation of proteins in juice was collected. The samples of DPJ were dried in hot air oven at 65^{o}C. The dry DPJ was stored in sealed glass jar until used. Sufficient care was taken to minimise absorption of moisture by the DPJ samples.

Preparation of culture media

The conventional gram negative (GN) medium was prepared by dissolving glucose 10 g, KNO_3 2.5 g, KH_2PO_4 1 g and $MgSO_4$ 0.5 g in one litre of distilled water. Simultaneously, the dry DPJ was dissolved in distilled water at various concentrations and used as a medium for growing fungi.

Sterilization

Twenty five ml of either GN medium or the aqueous solution of DPJ was poured into 250 ml conical flask. The flasks were then plugged with non absorbent cotton and autoclaved at 15 lbs for 30 minutes.

Inoculation

The autoclaved flasks were transferred to the inoculation room for inoculation with fungi. The stock cultures of the fungi were collected from the laboratory of department. It was maintained on potato dextrose agar (PDA) medium. The inoculation was always done in UV chamber under aseptic condition. The inoculum in the form of spore suspension was prepared by adding 10 ml sterile distilled water to six day old slope culture of the fungi. The medium, either GN or DPJ, was inoculated with 5 drops of the spore suspension which contained 5×10^2 spores per microscopic field. The inoculated flasks were incubated at room temperature.

Collection of microbial biomass

The flasks were inoculated for 8 to 12 days after inoculation. The fungal biomass was harvested by filtration through Whatman 1 filter paper. The mycelial biomass was dried along with the filter paper in an oven at 65 ± 5^{o}C till constant weight. The yield of mycelial dry weight (MDW) was then recorded by substracting the weight of filter paper from the weight recorded for dry mycelium.

A blank or control flask was also processed simultaneously, during all experiments wherein flasks containing either GN or DPJ medium remained uninoculated. The MDW was corrected each time by substracting the dry weight obtained from uninoculated flasks.

During all experiments, the culture filtrate was collected after harvesting the microbial biomass, centrifuged at 10,000 rpm for 15 minutes at 0^{o}C and supernatant was used as the crude enzyme extract as source of ∝ -amylase.

Assay of ∝-amylase

Amylase activity was determined using modified method described by Bern (1955). The starch solution (1%) for which was prepared by adding a paste of soluble starch to boiling phosphate buffer (20 mM) and stirring constantly with glass rod. This solution was filtered with Whatman paper No.1 before use.

Starch solution (1 ml) was taken in a glass tube and placed in water bath at 40°C for temperature equilibration. 1 ml properly diluted crude enzyme extract (either from GN media or DPJ) was added to pre-incubated starch. The reaction mixture was incubated at 40°C for 20 minutes and the reaction was terminated by adding 0.4 ml DNS reagent prepared by adding 1 g of 3,5 dinitrosalysilic acid (DNS) to 20 ml of 2 N NaOH and 30 g sodium potassium tartarate. The blank was prepared similarly but without substrate i.e. starch. The tubes were kept in boiling water bath after addition of DNS reagent for 5 minutes, cooled immediately under tap water and reaction mixture was diluted by adding 3 ml distilled water and the amylase activity was determined by measuring the optical density at 460 nm and comparing it with the calibration curve. The calibration curve was established with maltose (0.2 to 2 mg/2 ml water) and was used to convert the optical density into the amount of maltose released. Amylae activity was expressed in terms of mg of maltose liberated within one minute when the reaction mixture was incubated for 20 minutes at 40°C with 1 ml of the crude enzyme extract.

The unit of enzyme activity (U) was defined as the number of micromoles of ∝-1-4 glycosidic bonds hydrolyzed per minute and was calculated as described by using following equation:

$$\text{Unit (U)} = \frac{\mu\text{g of maltose equivalents produced/min/ml of digest}}{342}$$

Where, 342 is the molecular weight of maltose.

Production of ∝-amylase

During the first experiment, *A. niger, A. flavus, Helminthosporium oryzae, Fusarium oxysporum, Phytophthora infestans* and *Curvularia lunata* were grown on the DPJ expressed from *Medicago sativa, Anethum graveolens, Spinacia oleracea, Trigonella foenium-graecum, Coriandrum sativum* and also on conventional GN medium to evaluate the suitability of DPJ as a medium for fungal growth and subsequent production of enzyme ∝-amylase. The culture filtrates left after isolating the mycelium after 7 days of growth of these fungi were employed to assay ∝-amylase production as described above.

Effect of different flours on production of ∝-amylase

During present experiment, wheat, *Sorghum* and maize flours were added at different concentrations (5 to 50 mg/ml) to DPJ prepared from lucerne to enhance the production of ∝-amylase. Six fungi were inoculated to the DPJ enriched with flour and the yield of microbial biomass was recorded along with the measurement of ∝-amylase activity.

Results and Discussion

Deproteinised leaf juice, left behind isolating leaf protein concentrate from the heated juice is considered as a by-product of 'Green Crop Fractionation' system. As this product is rich in soluble nutrients from the plant its random disposal will not only cause environmental bio-pollution but also make the process of GCF inefficient or less economic. In view of its high cost stressed its proper use to make this process more valuable.

Earlier investigations from this laboratory indicated suitability of DPJ as a medium for growing useful fungi (Iliyas and Mungikar 2005) indicated suitability of lucerne DPJ for cultivating *A. niger* and production of ∝-amylase.

During present study, the efficiency of DPJ as a medium for fungal growth was evaluated and its value in microbial biotechnology for the production of ∝-amylase was tested.

Effect of different flours on ∝-amylase production

In microbial biotechnology, employed for the production of enzymes from fungi or other microbes, the composition growth medium is modified as per the requirement. It was observed that the DPJ from lucerne at a concentration of 2% is suitable for growing fungi. Though it is a rich source of nitrogen (N) for fungal growth the low level of carbohydrates limit further growth. Iliyas (2014) found increased growth of fungi when the DPJ was supplemented with either glucose or lactose (a source of carbohydrate). However, on large scale cultivation of fungi on DPJ the use of glucose may be expensive. In view of these attempts were made during present experiment to study the effect of wheat, sorghum and maize flours as a source of carbohydrate on the growth of various fungi and ∝-amylase production by them.

The results presented in Table 1-3 indicate that with the increase in concentrate of flour in the DPJ there was simultaneous increase in the mycelial dry weight (MDW) production of the six fungi under investigation. On DPJ alone, on an average, the yield of MDW was between 76.0 and 89.5 mg which increased to 164.5, 131.0 and 117.0 mg, respectively under the influence of the flours of wheat, sorghum and maize, respectively. Maximum response to the enrichment of DPJ by flour was noticed with *A. niger.*

Table 1. Effect of different concentrations of wheat flour on growth of fungi on DPJ of *Medicago sativa* L.

Fungi	Mycelial dry weight (mg)										
	mg/ml DPJ										
	0	5	10	15	20	25	30	35	40	45	50
Aspergillus niger	105	107	121	137	151	175	189	210	230	244	268
Aspergillus flavus	95	097	099	103	109	113	119	125	131	137	144
H. oryzae	98	105	109	111	120	125	131	137	145	155	169
F. oxysporum	91	097	101	110	117	121	126	132	135	140	143
Curvularia lunata	61	065	070	079	082	086	093	096	101	107	112
P. notatum	87	097	101	111	117	121	127	133	139	143	151
Mean	89.5	94.6	100.0	108.5	116.0	123.5	130.8	138.8	148.8	154.3	164.5
SD	13.9	13.8	14.2	16.9	20.1	26.4	28.8	34.5	39.7	42.6	49.2
CV	15.5	14.5	14.2	15.6	17.4	21.3	22.0	24.9	26.7	27.6	29.9

Table 2. Effect of different concentrations of Sorghum flour on growth of fungi on DPJ of *Medicago sativa* L.

Fungi	Mycelial dry weight (mg)										
	mg/ml DPJ										
	0	5	10	15	20	25	30	35	40	45	50
Aspergillus niger	90	093	099	101	117	121	129	134	144	150	161
Aspergillus flavus	98	101	117	121	125	130	135	140	143	145	149
H. oryzae	83	085	088	089	093	097	101	111	117	120	127
F. oxysporum	61	069	075	077	082	086	093	097	101	103	106
Curvularia lunata	68	073	077	081	085	087	089	093	094	099	101
P. notatum	99	101	102	107	109	113	117	121	125	129	137
Mean	83.1	86.7	93.0	96.0	101.8	105.6	110.6	116	120.6	124.3	13.1
SD	14.3	11.6	14.7	15.3	16.1	16.7	17.5	17.5	190.3	19.2	21.5
CV	17.2	13.4	15.8	15.9	15.1	15.8	15.8	15.0	15.7	15.2	16.5

Table 3. Effect of different concentrations of maize flour on growth of fungi on DPJ of *Medicago sativa* L.

Fungi	Mycelial dry weight (mg)										
	mg/ml DPJ										
	0	5	10	15	20	25	30	35	40	45	50
Aspergillus niger	87	090	103	107	113	117	123	129	131	134	141
Aspergillus flavus	93	095	097	101	105	107	111	113	117	121	125
H. oryzae	76	076	079	083	085	089	093	099	101	103	107
F. oxysporum	62	063	069	073	077	081	085	091	093	095	099
Curvularia lunata	78	085	089	095	101	111	117	121	125	129	121
P. notatum	60	063	069	071	076	081	086	091	095	097	099
Mean	76.0	78.6	84.3	88.3	92.8	97.6	102.5	107.3	108.1	113.1	117
SD	12.0	12.4	13.0	13.6	14.2	14.5	15.1	14.6	14.6	15.4	16.2
CV	15.8	15.8	15.5	15.4	15.3	14.9	14.7	13.6	13.5	13.7	13.8

Tables 4 to 6 give an account on $\propto$-amylase production under the influence of the flours of these three grains. As with the increase in growth of fungi with increasing concentration of flour, the activity of enzyme amylase also increased. On an average it was 34 to 96 U/ml when the fungi were grown on DPJ alone, which increased to 301, 265 and 276 U/ml under the influence of flours.

Table 4. Effect of different concentrations of wheat flour on production of amylase by fungi grown on DPJ of *Medicago sativa* L.

Fungi	Amylase activity (U/ml)										
	Conc. of flour (mg/ml DPJ)										
	0	5	10	15	20	25	30	35	40	45	50
Aspergillus niger	86	89	162	266	291	850	919	940	977	974	951
Aspergillus flavus	135	139	176	217	245	261	291	305	323	231	171
H. oryzae	57	61	89	135	160	167	167	208	220	236	229
F. oxysporum	20	22	34	36	116	174	199	185	181	144	144
Curvularia lunata	21	22	34	34	77	100	135	167	171	158	153
Penicillium notatum	66	68	80	139	144	153	162	229	275	283	158
Mean	64.1	66.8	95.8	137.0	172	284	312	339	357	337	301
SD	39.5	40.3	55.8	85.4	73.6	257	275	272	281	288	292
CV	61.6	60.3	58.3	62.0	42.8	90.6	88.3	80.3	78.9	85.6	97

Table 5. Effect of different concentrations of sorghum flour on production of amylase by fungi grown on DPJ of *Medcago sativa* L.

Fungi	Amylase activity (U/ml)										
	Conc. of flour (mg/ml DPJ)										
	0	5	10	15	20	25	30	35	40	45	50
A. niger	187	190	208	369	457	470	527	527	520	470	458
A. flavus	47	48	57	66	89	164	272	291	293	256	275
H. oryzae	160	185	190	229	270	323	360	366	382	366	380
F. oxysporum	77	89	121	137	171	178	194	213	227	203	181
C. lunata	89	94	130	139	213	227	236	254	231	185	185
P. notatum	21	23	38	89	91	135	139	178	190	182	116
Mean	96.8	104	124	171	215	249	288	304	307	276	265
SD	58.8	63.2	62.3	102	125	115	126	115	113	106	119
CV	60.8	60.3	50.2	59.4	58.3	46.2	43.9	37.9	36.8	38.7	45.0

Table 6. Effect of different concentrations of maize flour on production of amylase by fungi grown on DPJ of *Medcago sativa* L.

Fungi	Amylase activity (U/ml)										
	Conc. of flour (mg/ml DPJ)										
	0	5	10	15	20	25	30	35	40	45	50
Aspergillus niger	79	80	103	139	155	169	190	213	231	-	-
Aspergillus flavus	23	25	41	68	80	103	184	254	300	300	275
H. oryzae	17	34	43	139	167	190	227	233	282	587	300
F. oxysporum	48	59	75	89	121	167	245	254	222	158	135
Curvularia lunata	28	34	61	70	75	94	139	162	190	167	144
P. notatum	14	27	36	61	70	91	116	139	171	171	160
Mean	34.8	43.1	59	94.3	111	135	173	209	232	276	202
SD	22.5	19.8	23.4	32.6	38.9	40.5	48	44.2	46.0	163	70.0
CV	64.9	45.9	39.6	34.5	34.9	29.8	27.6	21.1	19.7	59	34.5

It was thus concluded that enrichment of DPJ with flours as a source of carbohydrate was useful in increasing the production of ∝-amylase. These flours might have contributed to the medium other nutrients apart from carbohydrates, like minerals, amino acids, vitamins etc. resulting in increased growth and enzyme production.

Conclusion

During the present course of investigation it is observed that suitability of DPJ as a medium for growing useful fungi. The results obtained during the experiments undertaken by author on DPJ for its potential as microbial growth medium indicated suitability of lucerne DPJ for cultivating *A. niger*. It was thus concluded from the experiments undertaken during present investigation that for maximum production of ∝–amylase take place by *A. niger*. The ∝–amylase production increased with the addition of wheat flour.

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References

Ansari SN and Iliyas S (2011). A comparative study of protein structure visualization tools for various display capabilities. Bioscience Discovery: An International Journal of Life Sciences, 2(2): 222-226.

Field BP (1955). Methods in Enzymology. (Colowick SP and Kaplan NO Eds.). Academic Press, New York Vol.1, 149 pp.

Iliyas S (2010). Effect of additives on chlorophyll content in wet lpc prepared from juice of Medicago sativa Linn. Bioinfolet-A Quarterly Journal of Life Sciences, 7(3): 251-259.

Iliyas S (2011). Study of LPC and PCR prepared from Radish (*Raphanus Sativus* Linn.) Plant Sciences Feed, 1(6): 88-92.

Iliyas S (2013). Production of amylase of DPJ of four different plants, International Journal of Basic and Applied Chemical Sciences, 3(4): 48-61.

Iliyas S (2014a). Effect of different flours on ∝-amylase production. American Journal of Scientific and Industrial Research, 5(3): 104-109.

Iliyas S (2014b). Production of α-amylase on deproteinised leaf juice prepared from different plants. Plant Sciences Feed, 4(4): 31-35.

Iliyas S and Ansari SN (2013). A comparative study of different properties provided by protein structure visualization tools. International Journal of Basic and Applied Chemical Sciences, 3(1): 1-6.

Iliyas S and Badar KV (2010). LPC: A good source of cyanocobalamine (B12), ascorbic acid (Vitamin C) and folic acid (Vitamin B9). Journal of Experimental Sciences, 1(2): 12-14.

Iliyas S and Badar KV (2010). Estimation of thiamine, riboflavin and pyridoxine from LPC of some plants. Journal of Experimental Sciences, 1(2): 15-17.

Iliyas S and Mungikar AM (2003). Changes in chlorophyll content of lucerne leaf juice during storage. Geobios, 30(4): 299-300.

Iliyas S and Mungikar AM (2005). Use of deproteinised leaf juice (DPJ) in microbial biotechnology. Pollution Research, 24(2): 459.

Josephin EDB and Iliyas S (2005). Deproteinised leaf juice as a medium for fungal growth and for production of protease. Geobios, 32(4): 282.

Sami P and Iliyas S (2014). A review on implementation of image processing in agriculture sector. Science Research Reporter, 4(1): 71-78.