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Research Article

EFFECT OF AMYLASE AND PROTEASE ENZYMES ON THE YIELDS OF LEAF PROTEIN CONCENTRATES PREPARED BY FERMENTATION OF LEAF JUICE

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ABSTRACT
The Leaf Protein Concentrate (LPC) can be prepared by various methods which include heat treatment, acidification, differential heat coagulation and natural fermentation. The protein content of LPC varied species to species by microkjeldahls method. The results during present studies suggested that heat coagulation of whole juice is the most suitable method for the preparation of leaf protein concentrate to be used in the human diet as protein supplement. The samples of leaf juice expressed during fractionation of *Vigna radiata*, Cowpea, *Dolichos* and byproduct leaves of cauliflower were fermented anaerobically for the preparation of LPC. The yields of LPC were recorded ; and the enzyme activities (amylase and protease) were measured in the DPJ samples by employing cup plate method. The case of cauliflower, the leaf juice was allowed to ferment for various periods, samples of DPJ collected and subjected to the measurement of amylase and protease enzyme activities. The yield of LPC declined due to the activities of enzymes amylase and protease during fermentation.

Keywords: *Leaf Juice, Fermentation, Leaf Protein Concentrate, Crude Protein, Amylase, Protease*

INTRODUCTION

Several methods for the preparation of LPC from leaf juice (Sayyed and Mungikar, 2003; Madhekar, 2008) have been advocated which include heating (Bagchi and Matai, 1976 ; Pirie, 1978; Carlsson and Clarke, 1983 ; Bhande and Mungikar, 1990), acidification and natural acidification due to anaerobic fermentation of juice (Shahane and Mungikar, 1988). The yield and quality of leaf protein concentrate change with the method by which it is prepared (Madhekar and Mungikar, 2009; Priscila, 2013). A major problem is gaining acceptability of leaf protein (LP) as a food residues in its colour and flavour (Roberto and Carlo, 1983). In order to obtain colourless or white LPC , fractionation of juice into cytoplasmic protein has been advocated. This is conveniently done by differential heat coagulation of juice at 60°C followed by coagulation at 95°C. The 60°C coagulum is largely chloroplastic protein which is dark green in colour ; while the 95°C coagulum is largely cytoplasmic protein which is white in colour (Chibnall, 1939). Recent investigations have shown that the green chloroplastic fraction is comparatively lower in protein content and contain more ash and lipids. On the other hand, the cytoplasmic fraction contain more protein and it is poor in lipid and ash content (Subba Rau *et al.*,1969 ; Knuckles *et al.*, 1975).

The process of Green Crop Fractionation (GCF) has been recommended for preparing high quality food grade product from green leaves (Pirie, 1971) . During GCF the leaf juice is extracted (Davys, et al., 1969) and heated to 90°C, as a result of which proteins in it coagulate to form leaf protein concentrate (LPC). The LPC is isolated from deproteinised juice (DPJ) portion by filtration through cotton cloth (Wadaskar *et al.*, 2015; Jadhav, 2018 b). The use of LPC in human nutrition as a source of protein and vitamin A is widely advocated (Telek and Graham, 1983 ; Sayyed , 2011).

It was observed during experimental phase that it is easy to separate heat coagulated LPC was coarse and posed problems during filtration. Furthermore, the acidification may prove costly in

comparison to heating and therefore the author feels that heat coagulated LPC could be a better alternative.

There is often a problem of acceptability of dark green LPC in human nutrition. To overcome this problem, Pirie (1971) suggested differential heat coagulation method which gives dark green chloroplastic LPC after heating the juice at 55°C and a yellowish white cytoplasmic LPC after heating the remaining portion at 95°C. The Chloroplastic LPC mainly contain enzyme proteins associated with photosynthetic process and are situated in chloroplast. This dark green LPC is rich in chloroplast as well as the two carotenoids xanthophyll and β –carotene (Sayyed and Mungikar, 2003 ; Badar and Sayyed, 2010). The cytoplasmic LPC mainly contains the enzyme, proteins and other soluble proteins which are present in the cytoplasm. It has been recommended that the green chloroplastic LPC in human nutrition . However, looking at the low yield of cytoplasmic fraction seems to be non practicable and it is felt that the process of GCF may prove uneconomical and inefficient if differential heat coagulation is adopted. (Jadhav and Mungikar, 2001)

An another alternative to either heat coagulation or acidification has been suggested by Stahman (1978). He suggested fermentation of juice, during which lactic acid is formed which acts as a natural acidification agent. When th juice is fermented in absence of oxygen, lactic acid is formed which decrease the pH of the juice and precipitate proteins. Earlier investigations in this laboratory, showed that fermentation of the juice results into decreased yields of LPC. However, during present investigation the yields of LPC prepared by fermentation remained almost at par to the heat coagulated LPC. This was probably due to the effect of season when the temperature was low enough. Fermentation of the juice have been recommended for the isolation of LPC . During fermentaton the carbohydrates in the juice are converted to lactic acid, as a result of which the pH decreases and results into coagulation of proteins to form LPC. Earlier studies in the laboratory by Kasture and Mungikar (1984) compared the process of making the LPC by heat coagulation and fermentation . They reported that during fermentation there is loss of dry matter as well as protein which results in decreased yield of LPC (Sayyed, 2010) .

During present investigation attempts were made to prepare LPC using the technique of anaerobic fermentation (Stahman, 1978). The leaf juice samples from *Vigna,* Cowpea and *Dolichos* were fermented for 6 days . Leaf juice obtained from cauliflower was fermented for various hours to study the yield of LPC. The DPJ left after the isolation of LPC from fermented juice was subjected for assay of hydrolytic enzymes amylase and protease (Sayyed and Mungikar, 2003 ; Jadhav, 2017).

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MATERIALS AND METHODS

The leaf juice samples from *Vigna*, Cowpea and *Dolichos* were fermented in conical flasks for 6 days . The curd of LPC resulting due to anaerobic fermentation was isolated for measurement of yield. The filtrate left after the isolation of LPC from fermented juice was subjected for the assay of enzyme amylase and protease by cup plate method.

120 ml of fresh juice of *Vigna*, Cowpea and *Dolichos* was placed in 125 ml of conical flask. The mouth of the flask was capped with rubber cork. A capillary rod was inserted through the cork and the mouth of the conical flask was sealed with wax. The flask was left at room temperature for fermentation of juice till 6 days. It was then filtered through whatman filter paper. Leaf juice obtained from cauliflower was fermented for 24, 48, 72, 96, 120, and 144 hours to study the yield of LPC. The filterates left after the isolation of LPC from fermented juice sample were subjected for the assay of enzymes amylase and protease by cup plate method (Gangawane and Mukadam, 1982; Jadhav, 2018a). The activity of enzyme was expressed as diameter of zone. The dry matter (DM) of LPC was determined by drying the samples in an electric oven at 95°C till constant weight (Jadhav, 2015) . The dried samples were ground to a

fine powder and stored in sealed glass containers for further analysis. Nitrogen (N) was expressed as N X 6.25 (Jadhav, 2014).

Enzyme assays : For amylase, soluble starch 10 gm, Na2HPO4 20 g, D.W. 1000 ml at pH 6.9. Autoclaved 15 ml medium was poured in petriplate. After solidifying the medium, cavity was made in centre.It is filled with culture filtrate and incubated. It is flooded with bugols iodine solution as an indicator.

For protease, the basal medium composed of 2% agar, 4% gelatin, 1% peptone,, 1% casein and pH was adjusted to 6.8. The enzyme activity zones were developed by covering the plates with 3% lead acetate. After washing, diameter of zones was measured (Jadhav, 2018 b).

RESULTS AND DISSCUSSION

Table 1 and figure.1, 2, 3, 4 and 5 illustrates on protein content on LPC samples prepared by various methods. The table indicates that the protein content varied from species to species and due to the method of its preparation. On an average all the LPC samples prepared by various methods yielded the product with desirable protein content (Dev et al., 1974 ; Deshmukh *et al.*, 1974 ; Kasture and Mungikar, 1984 ; Jadhav, 2015). The mean values indicates that, as compared with *Vigna*, Cowpea and *Dolichos* yielded more percent of crude protein content. The chloroplastic type of LPC by differential heat coagulation showed more percent crude protein content in *Dolichos* as compared with *Vigna* and Cowpea *i.e.*, 47.26 . Heat coagulation and acidification by H_2SO_4 also enhanced the percent crude protein in *Dolichos* as compared with *Vigna* and Cowpea, *i.e.,* 50.89 and 65.95 respectively. There was reduction of LPC percent crude protein by the process of fermentation of Dolichos leaf juice, *i.e.*, 34.29. In case cowpea, there was the enhancement of percent crude protein by fermentation of juice, i.e. 47.95. Acidification by HCL and cytoplasmic method of heat coagulation found beneficial to increase percent crude protein in LPC of Cowpea, *i.e.,* 62.62 and 32.62.

Table 1: Percent Crude protein contents of LPC samples prepared by various methods from *Vigna*, Cowpea and *Dolichos and its statistical analysis.*

No.	Methods of Preparing LPC	% Crude protein in dry matter of LPC		
		Vigna	Cowpea	*Dolichos*
1.	Differential Heat Coagulation			
	i) Chloroplastic	44.20	43.12	47.26
	ii) Cytoplasmic	27.20	32.62	32.35
2.	Heat coagulation	46.43	49.99	50.89
3.	Acidification with HCL	51.14	62.62	55.81
4.	Acidification with H_2SO_4	44.91	47.60	65.95
5.	Fermentation			
	Mean	*42.41*	*47.31*	*47.75*
	Standard Deviation	*8.20*	*9.75*	*12.84*
	Standard Error	*3.66*	*4.36*	*5.74*
	Coefficient of Variation	*19*	*21*	*27*

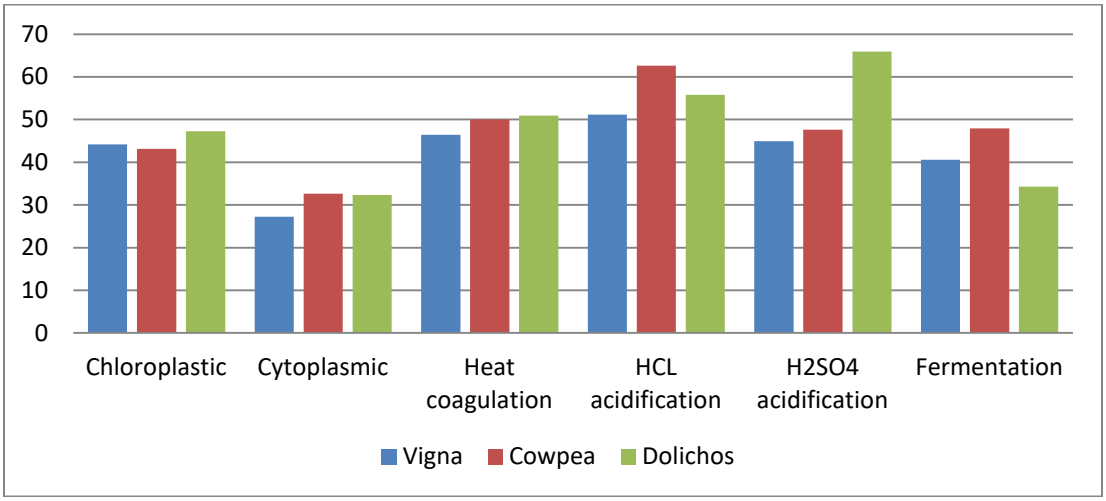

Figure 1: Crude protein contents of LPC samples prepared by various methods viz. chloroplastic, cytoplasmic, heat coagulation, HCL and H_2So_4 acidification and fermentation from *Vigna* , Cowpea and *Dolichos*

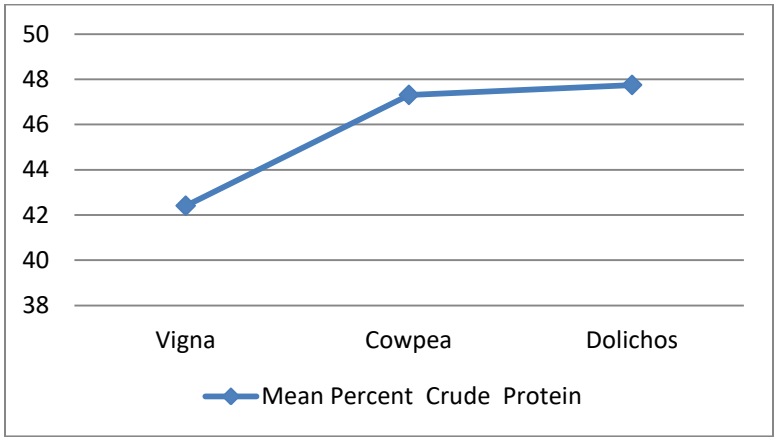

Figure 2: Statistical mean of percent crude protein by various methods of LPC preparation

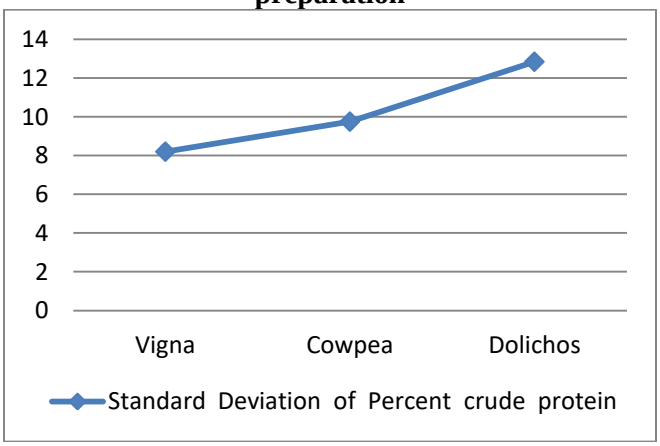

Figure 3: Standard deviation of precent crude protein by various methods of LPC preparation

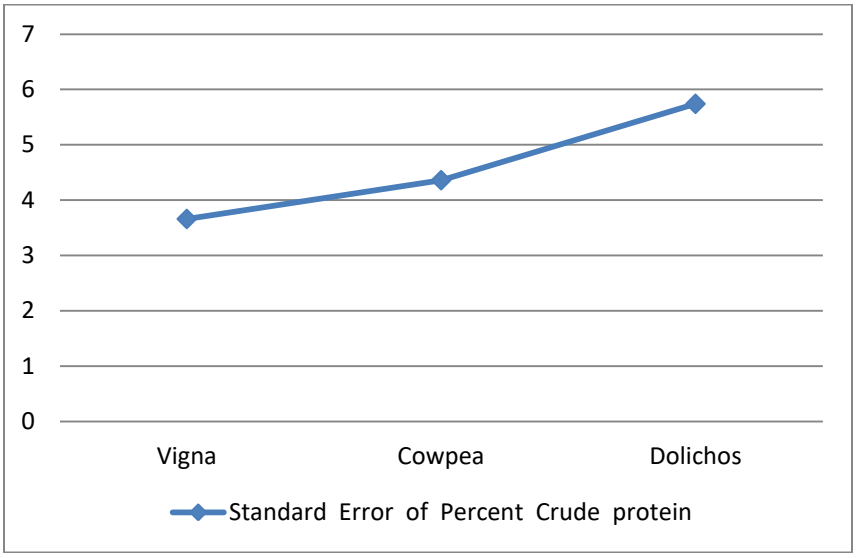

Figure 4: Standard Error among various methods of percent crude protein

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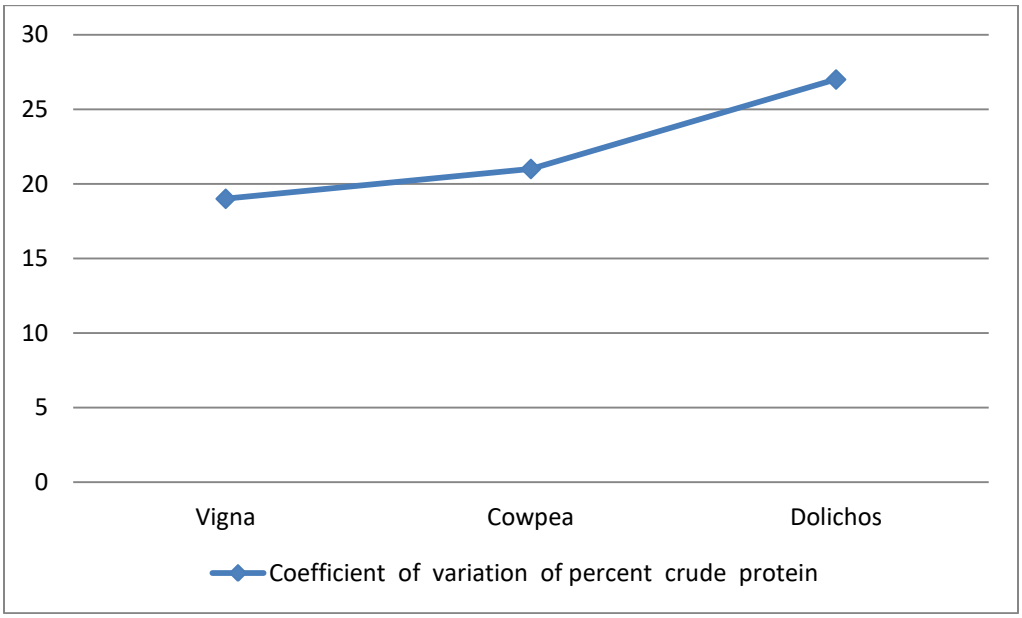

Figure 5: Coefficient of variation of percent crude protein

The results are presented in table 2. *Vigna* and Cowpea yielded more than 4 gm LPC while the yield of LPC from *Dolichos* was poor *i.e.,* 1,556 g (figure 6). In all juice samples amylase activity was prominent *i.e.*, 16, 20. and 16 mm respectively, (figure 8). The protease activity was minimum in cowpea, medium in *Vigna* and maximum in *Dolichos i.e.,* 12, 14 and 16 mm respectively. It can thus be concluded that during fermentation, the hydrolytic enzymes are secreted which breakdown carbohydrates and proteins resulting in lower LPC yields (figure 7).

Table 3: Yield and statistical analysis of LPC (g) , percent crude protein and the activities of enzymes amylase and protease zones (mm) in the deproteinised juice (DPJ) obtained after fermentation of leaf juices from cauliflower.

Fermentation Hours	LPC yield (g/120ml)	% crude protein	Diameter of Zone (mm)	
			Amylase	Protease
24	2.340	71	12	10
48	2.100	54.56	18	14
72	2.040	77.5	14	16
96	2.080	60.62	12	20
120	2.300	58.31	10	12
144	1.670	47.06	12	16
Mean	*2.088*	*61.50*	*13*	*14.66*
Std. Deviation	*0.23*	*11.8*	*2.75*	*3.50*
Std Error	*0.09*	*4.52*	*1.12*	*1.42*
C.V.	*11.44*	*18.01*	*21.15*	*23.87*

C.V.= Coefficient of variation

Table 3 indicates the effect of various hours of fermentation on the yields of LPC, percent crude protein and activity of enzymes amylases and proteases (Josephine and Sayyed, 2005). The results are clarified by the statistical analysis viz. mean, Standard deviation, Standard error and Coefficient of variation.

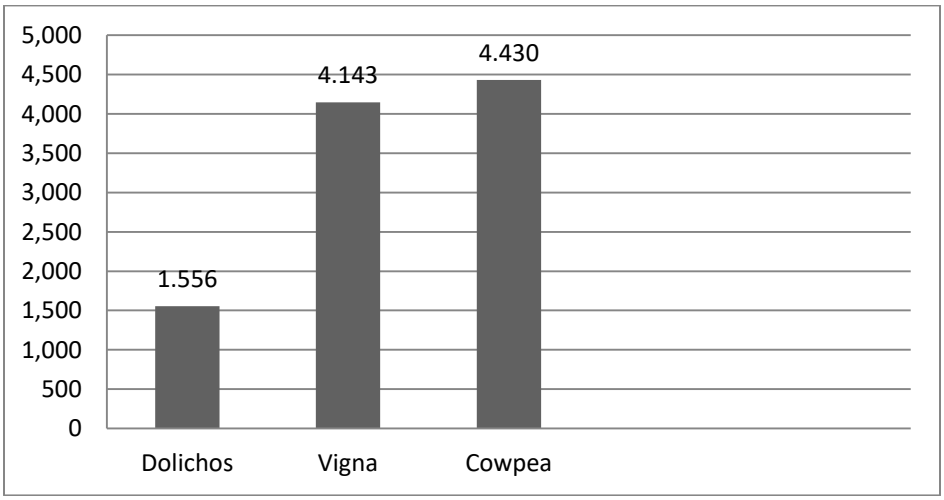

Figure 6: The yield of LPC in g/12 ml in *Vigna*, Cowpea and *Dolichos*

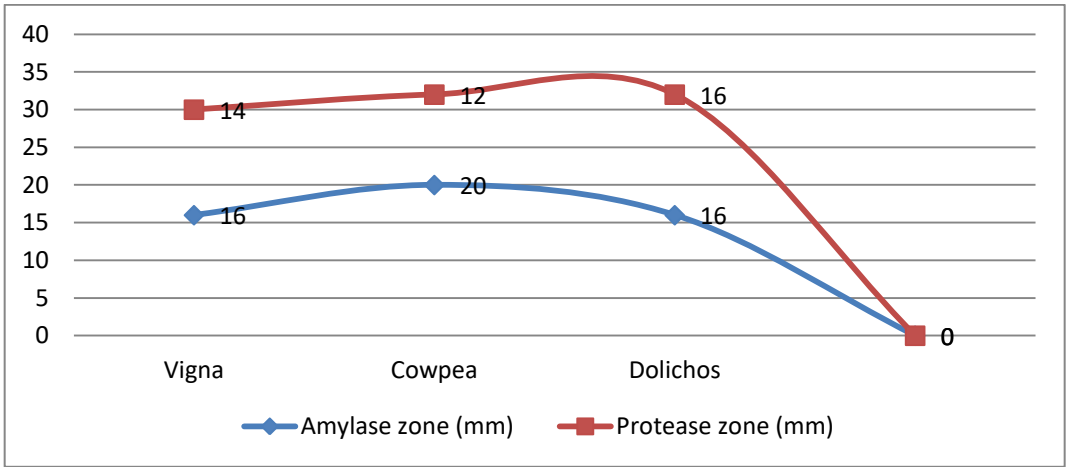

Figure 7: Enzyme zones (mm) of Amylase and Protease by cup plate method in *Vigna*, Cowpea and *Dolichos*

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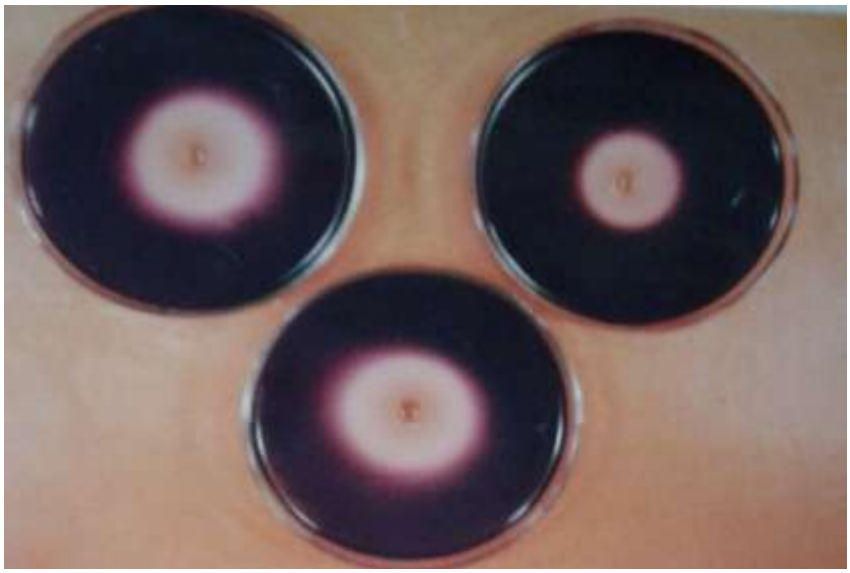

Figure 8: Enzyme activity of amylase by cup plate method by fermented juice of *Vigna*, Cowpea and *Dolichos* by cup plate method. The enzyme zone is formed by pouring iodine solution on agar medium with the starch as substrate. The amylase enzymes from fermented culture filtrates of juice from different leguminous leaves reacted with the substrate showing activity in the form of zone.

Leaf juice obtained from cauliflower was fermented for various hours, to study the yield of LPC and also to test activity of various enzymes, it was observed in cauliflower leaf juice, the yield of LPC decreased with the time of fermentation *i.e.*, 2.340 to 1.670 g, (figure 10). With respect to the amylase and protease enzyme production, the activity of the enzymes was high upto 96 hours after which it declined *i.e.* 12 to 10 mm and 13 to 16 mm respectively, (Table 3). Same in case of protein content, *i.e.*, 60.62 to 47.06 percent. The results indicate that amylases and proteases are produced in large amounts during earlier stages of fermentation (figure 12). At later stage a decrease in enzyme activity was observed, probably due to the change in pH and formation of new metabolic substances (figure 9).

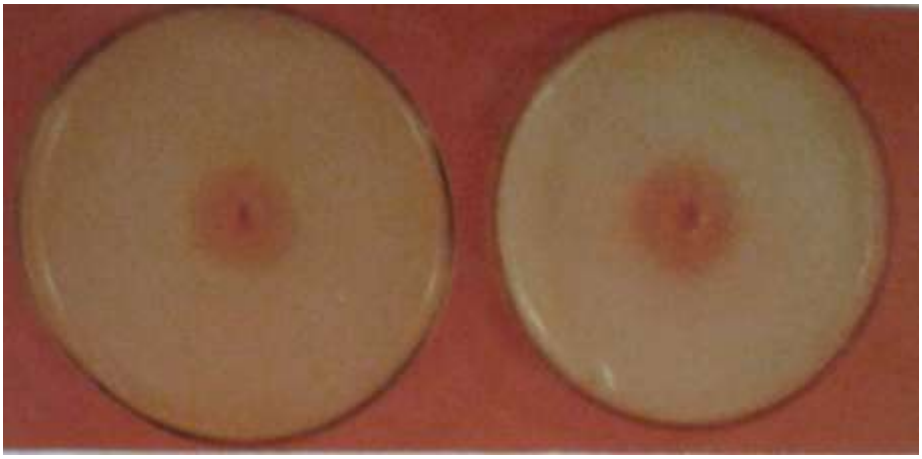

Figure 9: Enzyme activities of Protease by cup plate method by Cauliflower leaves fermented Juice DPJ after various hours.

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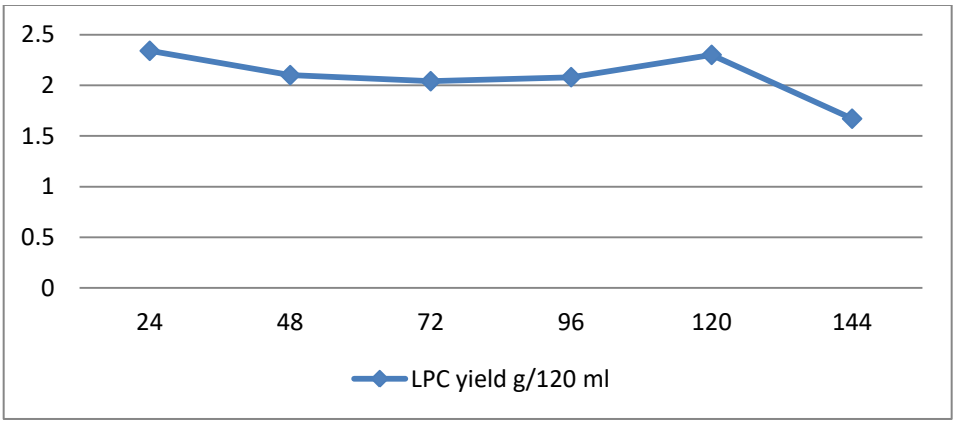

Figure 10: LPC yield g/120 ml in different fermentation hours of Cauliflower juice. (C) Percent crude protein contents of LPC byproduct obtained from fermented cauliflower leaf juice at various hours

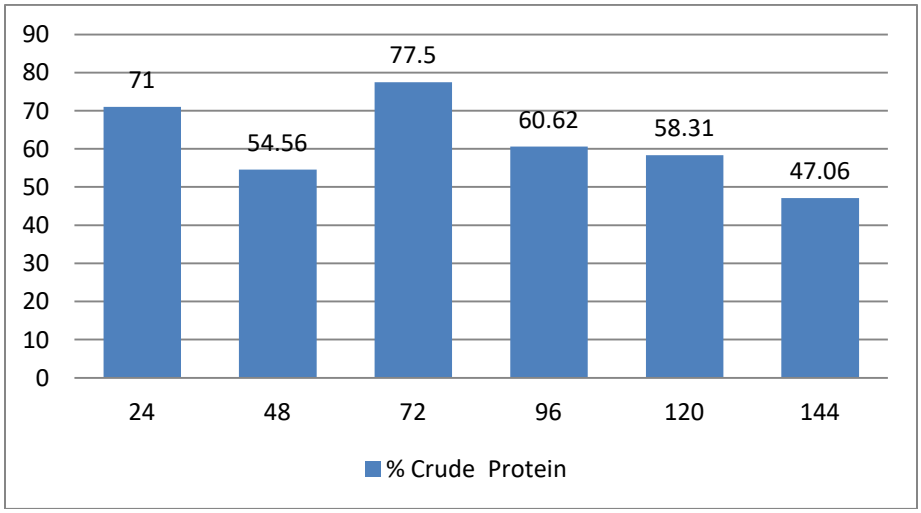

Figure 11: Percent crude protein contents of LPC byproduct obtained from fermented cauliflower leaf juice at various hours

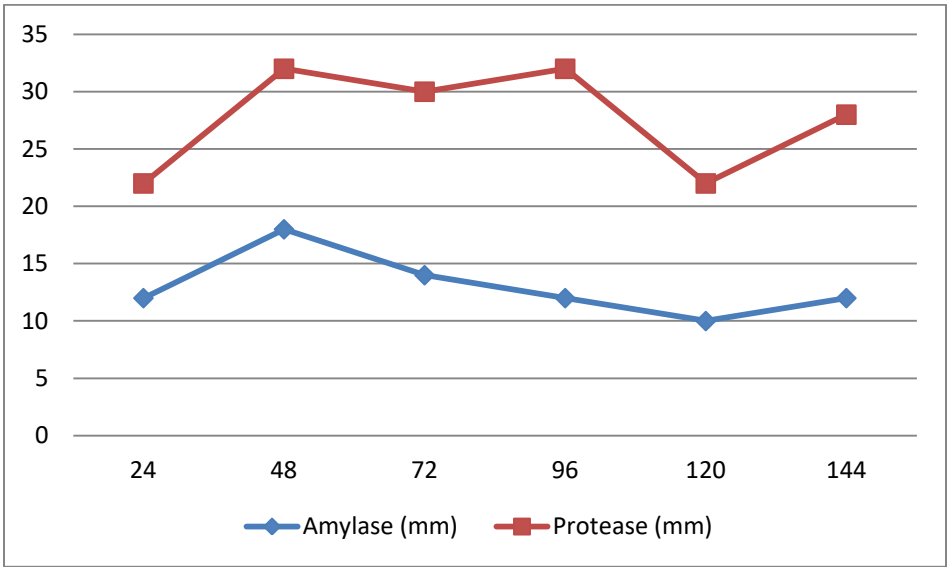

Figure 12: Diameter of enzymes amylase and protease activity zones by cup plate method by cauliflower leaf juice fermentation.

It is thus concluded that amylase and protease enzymes degrade starch and proteins in juice during fermentation, leading to lower yields of leaf protein concentrate (LPC) . There was decrease in percent of crude protein content due to fermentation (figure 11). Therefore other methods viz., heat coagulation and acidification are more conventional methods to prepare Leaf protein Concentrate (LPC) as compared with the fermentation method.

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Declaration of Conflict of Interest
The author declares that there is no conflict of interest.

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