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RESEARCH ARTICLE

Papaw fruit juice as source for single cell protein production using natural palmyrah toddy yeast

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Abstract: The study was carried out to perform Liquid State Fermentation (LSF) to produce Single Cell Protein (SCP) from papaw (*Carica papaya*) fruit juice using natural yeast obtained from Palmyrah (*Borassus flabellifer*) toddy. The LSF was performed in a shaking incubator (100 rpm) with the control fermentation medium (glucose) inoculated with 5 mL toddy. The glucose and $(NH_4)_2SO_4$ of the control medium were replaced with 100 ml/L of papaw fruit juice. The fermentation time, temperature and different concentrations of carbon and nitrogen sources on the SCP production were determined. After optimizing the conditions, the fermentation was carried out for 72 hours at 30°C with 5% of papaw fruit juice, and this has significantly increased the SCP yield to 40 % crude protein from 35.5 % (1.13 times). When the papaw juice was supplemented with inorganic nutrient supplements (KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$ and NaCl), the crude protein content was significantly ($p<0.05$) increased to 43.1 % from 40 % (1.08 times). After the reduction of nucleic acid using 1N NaOH, the crude protein yield increased further by 1 % (from 43.1 % to 44.1 %). When the medium was supplemented with nitrogen sources such as soybean flour, groundnut flour, corn flour, ammonium sulphate and peptone, no significant changes were determined. The amino acid analysis of the crude protein indicated that the product contained all the essential amino acids. Vitamin B analysis of SCP revealed that the crude protein contained thiamin (0.81 mg/100g) and riboflavin (2.3 mg/100g). Therefore, the production of crude protein from papaw fruit juice with natural toddy yeast culture could be used as a more economical method of producing SCP efficiently. This method can be further expanded in order to apply in industries.

Keywords: Liquid state fermentation (LSF), papaw fruit juice, single cell protein (SCP), palmyrah toddy, yeast.

INTRODUCTION

The ever increasing world population also requires an increase in food supply. The food protein shortage worldwide has become a concern at present. Increased protein demand led to the search for new and cheap protein supplements than conventional protein sources. Single Cell Protein (SCP) is one of the solutions suggested to meet the global protein demand. The SCP refers to dead, dry microbial cells which includes proteins, lipids, carbohydrates, nucleic acids, minerals and vitamins but most part of cell comprising proteins (Ware, 1977).

SCP has been produced from different microorganisms like algae, fungi, yeast and bacteria. These microorganisms utilize inexpensive carbon sources for the growth and production of bio protein. They also show the ability to upgrade low quality protein food into high quality protein food. SCP generally has high nutritive value due to higher content of proteins, vitamins and essential amino acids (Galvez *et al*., 1990). However, it may also have some nutritive issues like high nucleic acid content that may lead to slower digestion and some allergic reactions to humans (Anupama *et al*., 2000 and Ugbogu *et al*., 2016). If SCP is to be used successfully as a protein rich food, there are some criteria to be satisfied. Of which, the most important one being that SCP should contain high protein content with essential amino acids. Further, it should contain less than 2 % of nucleic acids and the production is economical. SCP has been produced from capsicum powder (Zhao *et al*., 2009), pineapple waste (Dhanasekaran *et al*., 2011), sugarcane bagasse (Rao *et al*., 2010 and Samadi *et al*., 2016), papaya extract (Maragatham *et al*., 2011), orange and cucumber peel (Mondal *et al*., 2012), cassava starch (Tipparat *et al*., 1995), wheat straw (Abou *et al.,* 1993), date waste (Mohamed *et al*., 2012), mango waste (Rashad *et al*., 1990), chinese potato (Anbuselvi *et al*., 2015), coconut waste water (Smith *et al*., 1976), banana waste (Saquido *et al*., 1981), apple waste (Khan *et al*., 2010) and vegetable oils (Mihaela *et al*., 2012). It is also known that organisms such as *Candida utilis, Saccharomyces cerevisiae, Penicillium janthinellum* and *Candida tropicalis* can produce SCP (Nasseri *et al*., 2011).

Papaw (*Carica papaya*) is an important fruit crop belonging to the family Caricaceae. Papaw is cultivated throughout the island and produce fruits year around. Papaw fruits contain high carbon content and less protein content (Miller *et al*., 1936). Palmyrah (*Borassus flabellifer*) toddy is a naturally available source containing diverse yeast species. Endogenous micro flora of toddy is not only composed of natural yeast but also contains less number of acetic acid and lactic acid producing bacteria (mixed-culture of toddy). The main aim of this study was to identify a cheap and an easy method of producing SCP using natural and cost-effective sources. In the study, papaw fruit juice and yeast of palmyrah toddy were used as

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the medium and the inoculums respectively to optimize the culture growing and fermentation conditions to increase the yield.

MATERIALS AND METHODS

Strains and media

Papaw fruits (variety- 'Red lady') were obtained from a local market at Thirunelvelly, Jaffna, Sri Lanka. Palmyrah toddy was directly obtained from a tapper during the period from March to May in 2017. Palmyrah toddy was collected from matured trees under required conditions (time – evening 4.00 -5.00 pm. and temperature 28 – 30^{o}C) using sterile vessels and this was used as the source of natural yeast.

Preparation of Papaw fruit juice

Ripened papaw fruits were washed with sterile water and peeled to remove the skin. Seeds were removed from sliced pulp and the pulp was macerated in a blender (National, MX-795N) for 5 minutes. The fruit juice was filtered with the use of a Muslin cloth. The juice extracted was placed in a steriled glass container and autoclaved at 121^{o}C and 15 psi for 15 minutes.

Preparation of the medium and fermentation

Papaw and control (Glucose) media were prepared according to the following composition (Control: D-Glucose - 10.0g , KH_2PO_4 - 1.0 g, $(NH_4)_2SO_4$ - 5 g, $MgSO_4.7H_2O$ - 0.5g, NaCl - 0.1g, $CaCl_2$ - 0.1g and distilled water 1,000 mL, Papaw based medium: papaw juice - 100 mL, KH_2PO_4 - 1.0g, $MgSO_4.7H_2O$ - 0.5g, NaCl - 0.1g, $CaCl_2$ - 0.1g and distilled water 900 mL).

Each medium was transferred into steriled bottles and autoclaved at 121^{o}C and 15 psi for 15 minutes. Then 50 mL of each medium was filled into six 250 mL Erlenmeyer flasks in triplicates. In each flask, 5 mL of natural toddy was added under sterile condition (microbial count $2.0405*10^6$ ± 2500 viable cells/1mL). The inoculated flasks were placed in a shaking incubator (JEIO TECH, SJ-600) at 100 rpm. After completion of each batch of fermentation, the fermented liquid was poured into centrifuge tubes and they were centrifuged at 4,000 rpm for 20 minutes (Mondal *et al*., 2012). Sediment was collected and oven-dried at 50 ^{o}C for 16 hours. Dry weight was measured and protein estimation was done according to the method of Kjeldahl (AOAC International.2006). Crude protein value was expressed as N*6.25.

Single cell protein production from papaw juice

Protein content of papaw fruit juice was initially determined using Kjeldahl method. Papaw only medium (papaw juice - 100 mL and distilled water 900 mL) was used in 250 mL of Erlenmeyer flasks. The flasks were inoculated with 5 mL of natural toddy under sterile conditions. Fermentation was allowed for 24 hours at 28^{o}C in a shaking incubator (100 rpm). Crude protein content was determined by Kjeldahl method.

Optimization of culture conditions for SCP production

Optimization of Fermentation time

Papaw medium (10 % concentration) was allowed to ferment with 5 mL of natural palmyrah toddy in Erlenmeyer flasks with three replicates (250 mL) at 28^{o}C in a shaking incubator (100 rpm). Samples were taken from the flasks after 1, 2, 3, 4, 5, 7 days of fermentation and the crude protein contents were determined using the Kjeldahl method (Samadi *et al*., 2016).

Optimization of fermentation temperature

Fermentation was carried out with papaw medium (10% concentration) at different temperatures such as 25, 28, 30, 35 and 40^{o}C using 5 mL of natural palmyrah toddy in Erlenmeyer flasks (250 mL). Fifteen samples were taken after three days of fermentation and the crude protein contents were determined.

Effect of different concentrations of carbon source in fermentation medium on SCP production

Fermentation was carried out with different concentrations of papaw sole medium (1, 2, 3, 4, 5, 10, 20, 50 and100% concentration of sole medium) at 30^{o}C using five mL of natural palmyrah toddy in Erlenmeyer flasks (250 mL). Twenty seven samples were taken after three days of fermentation and thecrude protein contents were measured (Babu *et al*., 2017).

Reduction of nucleic acid

Reduction of nucleic acid from microbial cells with NaOH was done (Herbert *et al*., 1971). 20 ml of 1 N NaOH was added to 5 g of dried microbial biomass. Removal of nucleic acid was performed in a boiling water bath for 10 minutes. After cooling to room temperature (28.5^{o}C), the solution was centrifuged and sediment was oven dried and the crude protein was determined.

Effect of different nitrogen sources on SCP production

Medium was added with different types of edible flour (corn flour, soybean flour, groundnut flour), $(NH_4)_2SO_4$ and peptone(5g/L)with papaw medium (5% juice concentration) and 5 mL of natural palmyrah toddy was added to Erlenmeyer flasks (250 mL) in triplicate and allowed for fermentation. Eighteen samples were taken after three days at 30^{o}Cand the crude protein contents were determined.

Determination of amino acid composition

Fermentation was carried out with 5% papaw juice medium at 30^{o}C using 5 mL of natural palmyrah toddy in Erlenmeyer flasks (250 mL). Samples were taken from shaking incubator after three days of fermentation and the protein yield was subjected for amino acid analysis. One mg of SCP biomass was hydrolyzed using 6N HCl for 22h at 110^{o}C and finally suspended in 1mM HCl (Fountoulakis *et al*., 1998). Amino acid composition was analyzed using an auto amino acid analyzer.

Determination of Vitamin B

SCP yield from the media after 3 days of fermentation was analyzed for Vitamin B composition such as thiamin and riboflavin using HPLC. Microbial dry biomass (2 g) was hydrolyzed using 0.1 N H_2SO_4 for 30 minutes at 121°C. Then it was adjusted to pH 4.5 with 2.5 M sodium acetate. After that 50 mg Takadiastase enzyme was added. Then, the mixture was filtered through a Whatman No. 4 filter and the filtrate was diluted with 50 mL of pure water and filtered again through a micro pore filter (0.45 µm). Twenty microliters of the filtrate was injected into the HPLC system. Quantification of vitamin B content was accomplished by comparison to vitamin B standards (Sami *et al*., 2014).

Cost analysis

Cost analysis was done in order to produce 100 gram of SCP from papaw fruit juice as a sole medium, addition of inorganic nutrients and after reduction of nucleic acid from SCP.

Statistical analysis

All experiments were carried out in triplicate and the graphs were expressed as mean value of the data. Experimental design was a Completely Randomized Design (CRD) and statistical analysis (ANOVA) was done using the SAS-8 statistical package. The means were compared using Duncan's Multiple Range Test (DMRT at 0.05α).

RESULTS AND DISCUSSIONS

Protein and nitrogen analysis of fresh papaw fruit juice

Protein and nitrogen contents of papaw fruit juice were determined. Amount of protein available in the papaw fruit juice was rather low (1.25 ± 0.065 %). Total nitrogen content was also low (0.2 ± 0.01%).

Single cell protein production from papaw juice

Crude protein content obtained after one day of fermentation at 28°C was 35.4 ± 0.12 %. This indicates that the toddy yeast could be used to produce relatively higher concentration of protein with papaw juice as the medium with no addition of other nutrient supplements. In order to increase the crude protein content, different culture growth conditions were used.

Optimization of culture conditions for SCP production

In order to increase the SCP content from papaw fruit juice, it was fermented using mixed culture of yeasts obtained from palmyrah toddy. The culture growing conditions such as fermentation time, incubating temperature, concentration of carbon source, reduction of nucleic acid and addition of different nitrogen sources to the fermentation media were optimized in order to improve the SCP production.

Fermentation time on protein production

Fermentation time is one of the key factors that influence the protein production within microbial cells (Jamal *et al*., 2008, Samadi *et al*., 2016). The result of this study is summarized in figure 1. Amount of crude protein produced was significantly higher on the 3rdday of fermentation (Control – 39.3 %, papaw medium - 42.0 %). Amount of crude protein produced showed an increasing trend with the increasing fermentation time from the first day to 3 days for both the media (control and papaw medium). Anyhow after 3 days of fermentation, crude protein content showed a reducing trend because of the limited availability of nutrients and reduction of carbon source in the medium during lengthened fermentation, probably impact on microbial cells to consume the produced protein (Samadi *et al*., 2016).Similar observations have been reported, in an experiment carried out with sugarcane bagasse using yeast (Samadi *et al*., 2016) and with pineapple waste using yeast (Dhanasekaran *et al*., 2011). In another study using *Candida utilis*, higher SCP yield was obtained after 54 hours of fermentation with Sabouraud glucose agar medium (Babu *et al*., 2017).

Fermentation time on biomass production

The dry biomass was increased with the increase in fermentation time. Biomass production was highest on the 7th day of fermentation in both papaw and control media (Figure 2). The biomass production through fermentation of papaw juice was significantly higher (0.51 g) than the control (0.389 g). Similar results were also obtained in an experiment carried out with sugarcane bagasse (Samadi *et al*., 2016) in a tray bioreactor and with pineapple waste using yeast as the inoculums (Dhanesekaran *et al*., 2011). Since the maximum crude protein yield was obtained on the 3rd day of the fermentation (Figure 1), the 3-day duration was selected as the fermentation time in further studies.

Effect of fermentation temperature

Temperature of the fermentation medium is one of the major parameters which affect the protein production in microbial cells (Walsh, 1977). Crude protein production was lower (Control – 36.3 %, papaw medium – 34.1 %) at 40°C whereas it was higher (Control – 40.3 %, papaw medium – 42.7 %) at 30°C (Figure 3). The optimum temperature for growth of yeast (*Saccharomyces cerevisiae)* had been reported to be active in the range of 30 - 35°C (Walsh, 1977). Therefore, 30°C was used as the temperature for further studies. Similar observation was noted by Babu *et al*., (2017) with Sabouraud glucose agar medium using *Candida utilis* at 30°C. At higher temperatures (at 40°C), SCP yield was lower and this may be due to the partial inactivation of enzymes which involved in metabolic reaction in microbial cells (Samadi *et al*., 2016).

Effect of concentration of carbon sources

Figure 4 depicts the influence of different concentrations of papaw juice as the sole medium for single cell protein production. The highest concentration of crude protein was obtained at 5% papaw juice. Babu *et al*., 2017 recorded the maximum protein content with *Candida utilis* at 5% sugarcane bagasse as the medium. The crude protein content increased with increasing fruit juice concentrations from 1% to 5%, followed by a decrease due to the substrate suppressive effect. The results suggest that organisms

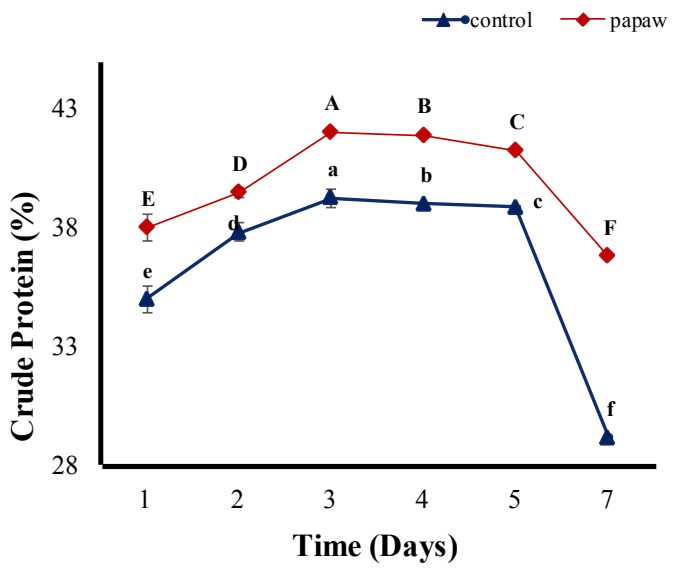

Figure 1: Effect of fermentation time on SCP production from toddy mixed culture at 28°C, under the liquid fermentation system. Mean significant differences were tested for control and papaw medium using DMRT at α level 0.05. Capital letters indicated significant difference between means of crude protein of papaw medium at different fermentation days. Small letters indicated significant difference between means of crude protein of control medium at different fermentation days.

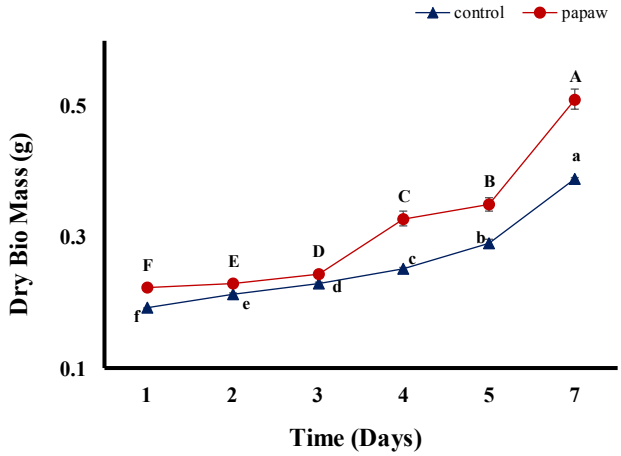

Figure 2: Effect of fermentation time on dry biomass production from toddy mixed culture at 28°C, under the liquid fermentation system. Mean significant differences were tested for control and papaw medium using DMRT at α level 0.05. Capital letters indicated significant difference between means of dry biomass content of papaw medium at different fermentation days. Small letters indicated significant difference between means of dry biomass content of control medium at different fermentation days.

found in toddy (yeast, lactic and acetic acid bacteria) are capable of grow in fruit juices without supplementation of inorganic nutrients, which could make SCP production more costly.

After the optimization (temperature, time and concentration of carbon sources), the amount of crude protein increased to 40% from 35.5 % (1.13 times). The amount of crude protein increased to 43.1% when other nutrient supplements were added except the nitrogen source (KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$ and NaCl) (Table-1). The results indicated that toddy microorganisms efficiently utilized inorganic nutrient supplements in order to produce a higher SCP. Similar observation was also recorded with orange and cucumber peel extracts with glucose supplemented fruit hydrolysates and yeast (Mondal *et al*., 2012).

Effect of reduction of nucleic acid for higher SCP production

Reduction of nucleic acids by the addition of 1N NaOH caused significant changes in the SCP production. When the nucleic acid was reduced using 1N NaOH, the amount of crude protein content was increased by 1 % (from 44.0% from 43.1 %) (Table 2). Similar observation was recorded with single cell protein produced by *Penicillium janthinellum* using sugarcane bagasse as the medium (Rao *et al.,* 2010). Increase of crude protein when the nucleic acid content decreased could be explained by the removal of protein adhered with nucleic acid.

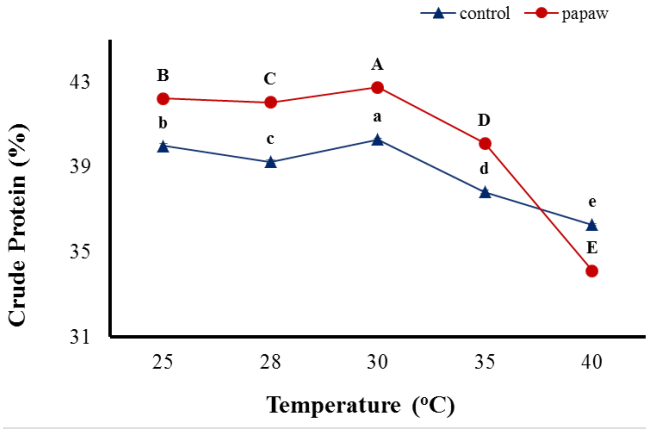

Figure 3: Effect of fermentation temperature on SCP production from toddy mixed culture in 3 days of fermentation time, under the liquid fermentation system. Mean significant differences were tested for control and papaw medium using DMRT at α level 0.05. Capital letters indicated significant difference between means of crude protein of papaw medium atdifferent temperatures. Small letters indicated significant difference between means of crude protein of control medium at different temperatures.

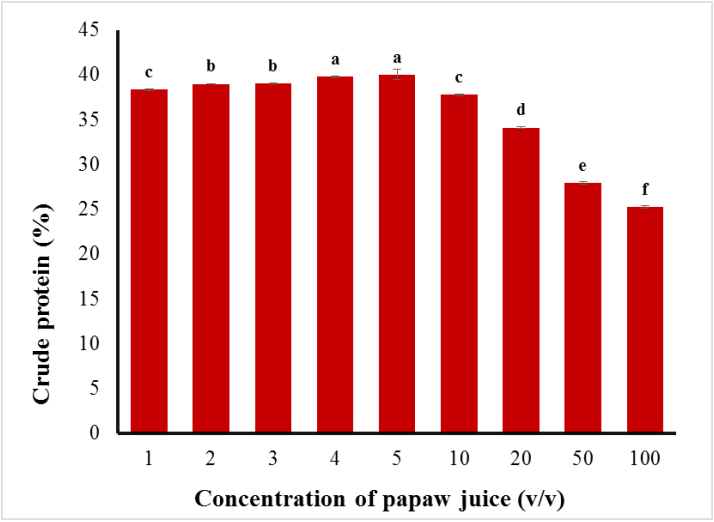

Figure 4: Effect of different concentrations of papaw juice sole medium on SCP production from toddy mixed culture in 3 days of fermentation at 30°C, under the liquid fermentation system. Mean significant differences were tested using DMRT at α level 0.05. Same letters indicated no significant differences between means.

Table 1: Crude protein content after optimization of culture growing conditions.

	Crude protein (%)	
	before optimization	**after optimization**
Papaya medium	40^{b}±0.076	43.12^{a}± 0.044

Values are the means ± standard error of 3 replicates.
Different superscript letters denoted significant difference between means of crude protein.

Table 2: Crude protein content after the reduction of nucleic acid from microbial cells.

	Crude protein (%)	
	before nucleic acid removal	**after nucleic acid removal**
papaw	43.12^{b} ± 0.044	44.05^{a} ±0.029

Values are the means ± standard error of 3 replicates.
Different superscript letters denoted significant difference between means of crude protein.

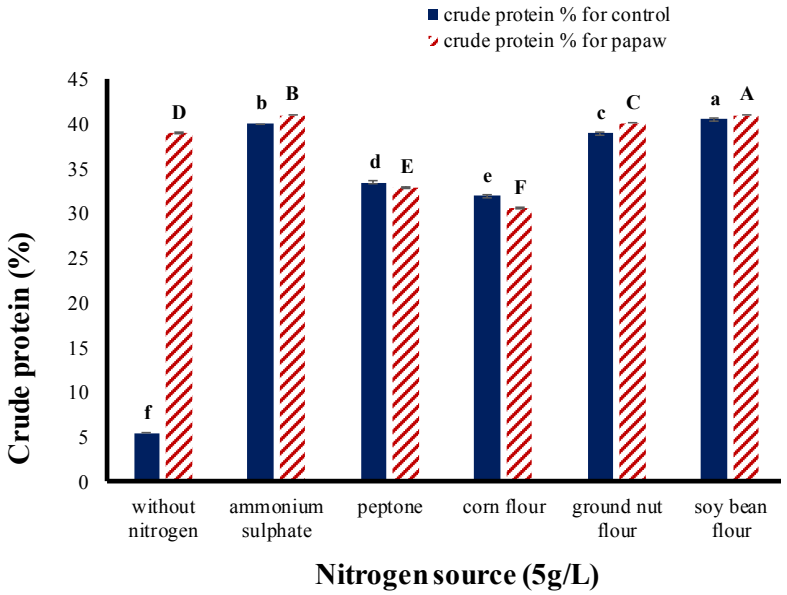

Figure 5: Effect of different nitrogen sources on SCP production from mixed culture of yeast for 3 days of fermentation at 30°C under the liquid fermentation system. Mean significant differences were tested for control and papaw medium using DMRT at α level 0.05. Capital letters indicated significant difference between means of crude protein of papaw medium at different nitrogen sources. Small letters indicated significant difference between means of crude protein of control medium at different nitrogen sources.

Effect of different nitrogen sources in fermentation medium on SCP production

The influence of nitrogen source on SCP production is given in Figure 5. When organic and inorganic nitrogen sources are added using locally available sources such as soybean flour, corn flour and ground nut flour, peptone and ammonium sulphate, the highest crude protein yield was obtained with soya bean flour (41.25%). In favour, the amount of crude protein produced was significantly low when corn flour was used (30.55%) in papaw juice. The SCP production showed no significant differences with different nitrogen sources as papaw juice itself contained enough nitrogen for microbial growth.

Amino acid composition

The amino acid composition of SCP produced from 5% liquid papaw fruit juice is summarized in the Table 3. The protein contains almost all the essential amino acids and some non-essential amino acids. Among these amino acids, methionine, arginine, valine and tryptophan were found in higher concentrations than the rest and phenyl alanine, threonine and lysine were relatively low. In comparison to FAO standards and soybean amino acids as a reference (Carrera *et al*., 2011), SCP contained a higher concentration of methionine. Lysine and valine contents were higher in the SCP produced by *Penicillium janthinellum* using sugarcane bagasse as the medium (Rao *et al.,* 2010). Amount of valine and tyrosine were higher than the other amino acids in the SCP produced from sugarcane bagasse using *Saccharomyces cerevisiae* (Samadi *et al*., 2016).

Vitamin B composition

The analysis of Vitamin B of SCP produced from 5% liquid papaw fruit juice revealed the presence of thiamin and riboflavin (Table 4). Other vitamin B components were either absent or at a trace levels in the extracted SCP. Thiamin and riboflavin were also reported in SCP produced from dextrose medium using *Candida tropicalis* (Chandrani *et al*., 2000).

Following the optimization of the culture growth conditions and media composition and the reduction of nucleic acids from microbial cells, the SCP production was increased from 35.45 to 44.05% (1.243 times) in the present study.

Cost Analysis

When only papaw fruit juice was used as the medium, the cost for the production of 100 g SCP was LKR 108.00, indicating a cost effective method of SCP production using papaw fruit juice and Palmyrah toddy. Therefore, SCP can be effectively used as a protein supplementation in pace of high cost conventional protein foods. When papaw medium is supplemented with other nutrients (NaCl, $CaCl_2$ and $MgSO_4$), the cost for 100 g SCP was LKR 128.00. The reduction of nucleic acid from SCP could increase the production costs by 1.22 times.

Table 3: Amino acids composition (% dry matter) obtained from SCP produced from papaw fruit juice as mediumduring auto amino acid analysis.

Amino acids	Amount (% dry matter)	Soybean	FAO standard
Lysine *	0.2[c]	2.37[b]	4.2[a]
Tryptophan *	2.2[a]	-	-
Threonine*	0.1[b]	-	2.8[a]
Methionine *	3.2[a]	0.59[c]	2.2[b]
Valine *	2.2[b]	1.94[c]	4.2[a]
Isoleucine *	1.1[b]	1.97[a]	-
Leucine *	1.8[c]	3.47[b]	4.8[a]
Glutamate	0.6[b]	8.14[a]	-
Phenylalanine*	0.1[c]	2.25[b]	2.8[a]
Histidine*	0.9[b]	1.05[a]	-
Arginine	2.4[b]	2.91[a]	-

Essential amino acids are denoted as *
Different superscript letters denoted significant difference between means of particular amino acid content row wise.

Table 4: Thiamin and Riboflavin content of SCP from papaw juice (mg/100g).

	Papaw medium
Thiamin	0.81
Riboflavin	2.3

Table 5: Cost of 100 g SCP production from papaw fruit juice medium.

	Papaw (Rs.)
Price of 1 kg fruit	80
Cost for 100 g SCP production from 1 kg fruit	83
Other charges including Palmyrah toddy and labour cost for 100 g SCP production	25
Total cost to produce 100 g SCP from sole papaw juice medium	108
Cost of inorganic nutrients (NaCl, $CaCl_{2,}$ $MgSO_{4)}$	20
Cost of NaOH to reduce nucleic acid content from SCP	28
Total cost for 100 g SCP production after addition of nutrients in the medium and reduction of nucleic acids	156

CONCLUSION

The present findings revealed that papaw fruit juice and natural toddy can be used as an effective combination of a carbon source and an inoculants to produce SCP containing all essential amino acids and some of vitamin B components including thiamin and riboflavin.

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