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ORIGINAL PAPER

Characterization of composition, antioxidant potential and microbial organisms upon submerged *Cicer arietinum* fermentation

Ahmed M. Saad[1] · Ragab A. Elmassry[1] · Ahmed Saaed Hamed[1] · Khaled M. M. Wahdan[1] · Mohamed Fawzy Ramadan[1,2]

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Abstract The aim of this work was to investigate the composition, antioxidant potential and microbial content of chickpea (*Cicer arietinum*) steep liquor (CSL) during a submerged fermentation. Chickpea seeds (250 g) were soaked in boiled distilled water (1:2, w/v) for 24 h at 37 °C then filtered and freeze-dried to obtain 8.2 g of CSL. Lysine was the main amino acid accounted for 77.0 % of total free amino acids followed by serine (6.49 %). The results of total amino acids found in CSL indicated that arginine was the main amino acid accounted for 24.0 % of total amino acids followed by tyrosine (20.0 %). Total carbohydrate in the freeze-dried CSL was 1.47 %, wherein reducing sugar was 1.25 % of total carbohydrates. Levels of nicotinic acid, pyridoxine, thiamin, riboflavin, folic acid, and vitamin B_{12} were 14.3, 3.14, 24.2, 1.11, 0.59 and 24.5 mg/100 mg CSL, respectively. CSL exhibited antioxidant activity (AA) wherein AA was increased with increasing the fermentation time. After 24 h of fermentation, AA of CSL reached 77.0 % while tertiary butyl hydroquinone exhibited 82.0 %. Twenty-five Bacilli isolates were separated from freeze-dried CSL on nutrient broth medium. CSL might be used as an alternative to yeast extract for syngas fermentation because it is rich in nutrients and cheaper compared to yeast extract.

Keywords Syngas fermentation · Amino acids · Steep liquor · Chemical composition · Microflora

✉ Mohamed Fawzy Ramadan
hassanienmohamed@yahoo.com

[1] Department of Agricultural Biochemistry, Faculty of Agriculture, Zagazig University, Zagazig 44519, Egypt

[2] Deanship of Scientific Research, Umm Al-Qura University, Mecca, Kingdom of Saudi Arabia

Introduction

Chickpea (*Cicer arietinum*) is one of the oldest and most widely consumed legumes in the world. Chickpea seeds are a cheap source of high quality protein, carbohydrates, and minerals in the diet. Chickpea is considered a healthy vegetarian food and it is one of the most important human foods [1]. Chickpea cultivars were grouped into two types: desi (Indian origin) and kabuli (Mediterranean and Middle Eastern origin) [2]. Differences in those two groups were observed with regard to their seed coat percentage, crude fiber content, trace element composition, polyphenol content [3, 4], parching properties [2], and properties of their flours [5].

Chickpea has also some medicinal benefit [6, 7]. Chickpea is also utilized in either whole or paste form as a main or side dish after cooking [8] or as a snack food after roasting [9]. It is a rich source of lysine and together with carbohydrates and minerals offers nutritional benefits when added to bread formulations [10]. In some Mediterranean countries, fermented chickpea is being used as a leavening agent to make traditional bread. Adding fermented chickpea in the wheat flour enhanced the nutritional quality and expanded the shelf life [11].

Steep liquor of some plants such as corn steep liquor is an important, cheap and environmental-friendly in many products. The development of an alternative low cost fermentation medium containing essential nutrients required for cell growth would reduce the cost of the fermentation process. Standard medium for *Clostridium* strain P11 is composed of yeast extract (YE), vitamins, minerals, trace metals and reducing agent [12, 13]. Apart from the reducing agent, YE is the most expensive component. Some cheap nutrients that could replace YE are corn steep liquor, hydrolyzed cottonseed flour, hydrolyzed soy flour and ethanol stillage [14].

Changes in enzyme activities and in the chemical composition of the fermented liquid are caused by indigenous bacilli and clostridia populations [15]. Changes in the chemical constituents of the fermented liquid can be attributed to *bacilli* until 8–10 h of fermentation then to clostridia until 18 h. *Bacillus subtilis* are important starter cultures for alkaline fermentations observed in traditional fermented legumes, such as the Indian Kinema (from spontaneously fermented soybeans), the African Soumbala (from locust beans) and others fermented legumes [16]. During fermentation of chickpea extracts, a significant decrease of pH was observed [15]. Similarly, during Gergoush-making from milk, lentils, and wheat flour, the dominant bacteria were bacilli, clostridia and lactic acid bacteria, and the pH of the fermenting product was reduced [17].

Hatzikamari et al. [15] reported that during chickpea fermentation, reducing sugars were increased while an increase in the free amino acids was observed. Since the reducing sugars support the growth of indigenous flora, they were reduced by 50 % after 18 h of fermentation due to their consumption by increasing population of clostridia. The degradation of chickpea proteins became obvious after 8–10 h of fermentation; presumably, due to the proteolytic activities of *Bacillus* spp. The antioxidant activity of soybean was clearly related to the amino acid composition [18]. It was reported that an increase in hydrophobicity of peptides would increase their solubility in lipid and therefore enhance their antioxidant activity. It was commonly believed that some amino acids such as histidine (His), methionine (Met) and cysteine (Cys) are very important to the radical scavenging activity of peptides due to their special structures of characteristics. For example, the imidazole group in His has the proton-donation ability; Met is prone to oxidation of the Met sulfoxide while Cys donates the sulfur hydrogen.

The aim of this work was to investigate the chemical composition, antioxidant potential and microbial content of chickpea liquor during fermentation.

Materials and methods

Materials

Chickpea (*C. arietinum*) were purchased from local market in Zagazig city (Egypt). Seeds were hand-sorted to remove wrinkled, moldy seeds and foreign material then stored in polyethylene bags in the refrigerator (4 °C) until needed. Tryptic Soy Agar was purchased from Biolife (Milano, Italy). DPPH˙(1,1-diphenyl-2-picrylhydrazyl radical), isopropanol, hydrochloric acid and sodium hydroxide were obtained from Merk (Germany). All chemicals used in the experiments were of analytical grade.

Methods

Preparation of chickpea steep liquor (CSL)

Chickpea seeds (250 g) were submerged and soaked in 500 mL boiled distilled water (1:2, w/v) for 24 h at 37 °C according to Badshah et al. [19]. After 24 h, foam was formed and the beaker was taken from incubator. In another 500 mL beaker, filtration was run using Whatman paper No. 1 and about 300 mL of CSL was recovered.

Preparation of hydrolyzed chickpea steep liquor (HCSL)

HCl (2.5 %) was added to 50 mL CSL in an oven at 100 °C for 12 h then the hydrolysate was filtered using Whatman No. 1 paper to obtain hydrolyzed chickpea steep liquor's (HCSL) with pH 1.3. pH was adjusted using 2.5 % NaOH to 4.7. HCSL was filtered and the filtrate pH was adjusted again to pH 7 using 2.5 % NaOH. HCSL and CSL were freeze-dried using lyophilizer (Thermo Fisher).

Composition of CSL

Sugars The CSL was extracted with 80 % ethanol, centrifuged at $2200\times g$ and the amount of reducing sugars were determined according to Miller [20]. Three mL of the clear ethanol extract were mixed with 3 mL of 3,5-dinitrosalicylic acid reagent (3,5-dinitrosalicylic acid 1.0 % w/v in 1.0 % w/v NaOH, 0.2 % w/v phenol and 0.05 w/v Na_2SO_3 was added just before the use), boiled at 95 °C for 15 min, then 1 mL of 40 % (w/v) potassium-sodium tartrate solution was added. After cooling, the absorbance was measured at 575 nm. A standard curve was made with known concentrations of glucose. The amount of reducing sugars finally expressed as mg of glucose/mL of CSL, while total carbohydrates were determined according to Hedge et al. [21].

Vitamins *B vitamins* are a group of water-soluble *vitamins* that play important roles in cell metabolism. Vitamins were determined using HPLC according to the method of Batifoulier et al. [22]. The liquid chromatography system consisted of Alliance 2960 Separation Module (Waters, Saint Quentin en Yvelines, France) with a Multi λ fluorescence detector (Waters 2475). The column (15 cm × 4 mm) and the pre-column (2 cm × 4 mm) were packed with a RP-amide C16 stationary phase with a particle size of 5 µm (Supelco, USA). The column and the guard column placed in an oven at 30 °C. The mobile phase was potassium phosphate buffer (50 mM, pH 6). Methanol (80/20, v/v) delivered at flow rate of 1 mL/min. The injection volume was 20 µL and the duration of the analytical run was 10 min. Fluorescence detection operated at 366 nm excitation and 435 nm emissions.

Amino acids Free and total amino acids were determined in CSL and HCSL using an INGOS Ltd AAA 400 automatic amino acid analyzer. Acid hydrolysis was carried out according to method of Block et al. [23]. The freeze-dried sample (100 mg) was hydrolyzed with 6 N HCl (10 mL) in a sealed tube at 110 °C for 24 h. The excess of HCl was then freed from 1 mL hydrolyzed under vacuum with occasionally addition of distilled water, then evaporated to dryness. The HCl free residue was dissolved in an extract (2 mL) of diluting buffer (pH 2.2). The buffer was used for dilution of samples and standards to the required concentration.

Antioxidant activity

DPPH· radical scavenging activity This assay was performed to study the effect of soaking time on the antioxidant potential of CSL. 10 g of chickpeas were submerged soaked in 20 mL boiled distilled water and immediately filtrated on Whatman paper No. 1 to obtain 10 mL of CSL. The experiment was repeated at time intervals of 8, 16 and 24 h at room temperature (22 °C) and at 37 °C.

The electron donation ability of a various fermented stages of CSL was measured by bleaching of the purple colored solution of DPPH· according to the method of Hanato et al. [24]. Three hundred µL of each submerged liquor (300 mg extract/1 mL solvent) was added to 3 mL of 0.1 mM DPPH· dissolved in methanol. After incubation period of 30, 60 and 120 min at room temperature, the absorbance was determined against a control at 517 nm [25]. Percentage of antioxidant activity of free radical DPPH· was calculated as follow:

$$\text{Antioxidant activity (inhibition)}\% = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100$$

where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of CSL. TBHQ was used as a positive control. Samples were analyzed in triplicate.

β-Carotene-linoleic acid bleaching The ability of CSL and synthetic antioxidant (TBHQ) to prevent the bleaching of β-carotene was assessed as described by Keyvan et al. [26]. In brief, 0.2 mg of β-carotene in 1 mL of chloroform, 20 mg of linoleic acid and 200 mg of Tween 20 were placed in a round-bottom flask. After removal of the chloroform, 50 mL of distilled water were added and the resulting mixture was stirred vigorously. Aliquots (3 mL) of the emulsion were transferred to tubes containing CSL or TBHQ. Immediately after mixing 0.5 mL of extract solution (300 mg extract/1 mL solvent), an aliquot from each tube was transferred to a cuvette and the absorbance at 470 nm was recorded (Abs^0). The remaining samples were placed in a water bath at 50 °C for 2 h then the absorbance was recorded (Abs^{120}) at 470 nm. A control without extract was also analyzed. Antioxidant activity was calculated as follows:

$$\text{Antioxidant activity } (\%) = \left[1 - \left(\text{Abs}^0_{\text{sample}} - \text{Abs}^{120}_{\text{sample}}\right)\Big/ \left(\text{Abs}^0_{\text{control}} - \text{Abs}^{120}_{\text{control}}\right)\right] \times 100$$

where $\text{Abs}^0_{\text{sample}}$ is the absorbance of sample at 0-time, $\text{Abs}^{120}_{\text{sample}}$ is the absorbance after 120 min, $\text{Abs}^0_{\text{control}}$ is the absorbance of control at 0-time, and $\text{Abs}^{120}_{\text{control}}$ is the absorbance of control after 120 min.

Isolation and identification of microorganisms

A medium used to determine the total bacterial count consisted of peptone (5.0 g/1000 mL water), beef extract (3 g/1000 mL) and agar (20 g/1000 mL) wherein the pH was adjusted to 7.0 [27].

One gram of freeze-dried CSL was suspended in 9 mL sterilized water in conical flask (100 mL), thoroughly shacked for 10 min and serial dilution series up to 10^{-7} were prepared. Plates incubated at 30 °C for 48 h then individual colonies picked up and the purified isolates maintained on nutrient agar at 4 °C. Isolates were counted according to Dilution-plate count where total count, spore formers, total proteolytics and total saccharolytics were incubated at 30 °C for 2–3 days on plate count agar, milk agar and glucose agar supplemented with bromocresol purple [28, 29].

Acid gas production test was performed according to the method described by Seely and Vandemark [30]. Bacterial isolates were tested for acid and gas production by inoculating 5 mL of the sterile glucose broth with bromocresol purple (15 mL/L of 0.04 % solution as pH indicator) in test tubes containing Durham's tube. The tubes were incubated for 7 days at 30 °C. Accumulation of gas in Durham's tube was taken as positive for gas production. The change in color of the medium to yellow was taken as positive for acid production.

Casein hydrolysis was performed according to Seely and Vandemark [30]. Petri plates of skim milk agar were streaked with test cultures and incubated at 30 °C for observing clear zone against black background.

Bergey manual and Biolog system number 21124 Cabot Blvd. Hyward CA 94545 (2004) in Cairo MERCIN was used to identify the most active isolates according to Klingler et al. [31].

Statistical analysis

All measurements were carried out in duplicate. Data obtained were subjected to analysis of variance (ANOVA) and Duncan's test using the software program of Statistical

Package for the Social Sciences (SPSS, edition 16.0), in order to assess significant differences among samples. Differences were considered significant when $p < 0.05$.

Results and discussion

Chickpea, one of the oldest and most widely known legumes, stands as a promising raw material for the preparation of fermented food products. Fermentation brings desirable changes in legume seeds, such as elimination of off flavors, improvement in digestibility, and enhancement in keeping quality and safety as well as better nutritional value. Results indicated that 8.2 g of freeze-dried CSL were obtained from 250 g of chickpea (3.28 %, w/w) after 24 h of fermentation at 37 °C.

Composition of CSL

The free amino acid content of the CSL was recorded using amino acid analyzer. Table 1 represents the results of free amino acids found in CSL after 24 h. Lysine was the main amino acid accounted for 77.1 % of total free amino acids followed by serine (6.49 %). Other amino acids were found in lower amounts. Data in Table 1 refer also to the results of total amino acids found in CSL after 24 h fermentation. Argenine was the main amino acid accounted for 24.3 % of total amino acids followed by tyrosine (20.1 %). Other amino acids were found in lower levels or in traces.

Total carbohydrate in the CSL after 24 h fermentation was 1.47 % w/w, while the total reducing sugar was 1.25 %. The B vitamins were once thought to be a single vitamin, referred to as vitamin B and they are chemically distinct vitamins that often coexist in the same foods. In general, supplements containing all eight compounds are referred to as a vitamin B complex. In general, the contents of thiamin, riboflavin, and niacin were increased during the fermentation according to [32]. Individual B vitamin supplements are referred to the specific name of each vitamin (e.g. B_1, B_2 and B_3). The obtained results indicated that the levels of nicotinic acid, pyridoxine, thiamin, riboflavin, folic acid, and vitamin B_{12} were 14.3, 3.14, 24.2, 1.11, 0.59, and 24.5 mg/100 mg CSL, respectively.

Antioxidant properties of CSL

Antioxidants react with $DPPH^{\cdot}$, reducing the number of $DPPH^{\cdot}$ free radicals to the number of their available hydroxyl groups. Therefore, the absorption at 517 nm is proportional to the amount of residual $DPPH^{\cdot}$ [33]. It is visually noticeable as a discoloration from purple to yellow. The scavenging activity of the tested extracts against $DPPH^{\cdot}$ was concentration-dependent. The results of $DPPH^{\cdot}$ radical scavenging activities of various fermentation stages of CSL are represented in Fig. 1. The results clearly indicated that CSL exhibited antioxidant activity (AA). In general, AA of CSL increased with increasing the fermentation time. After 24 h fermentation, AA of CSL reached 77 % while TBHQ exhibited 82 %.

It has been proven that the AA of various fermentation stages of CSL is mainly ascribable to the concentration of phenolic compounds in the plant [34]. The results of the $DPPH^{\cdot}$ free radical scavenging assay suggest that components involving CSL are capable of scavenging free radicals via electron- or hydrogen-donating mechanisms and thus might be able to prevent the initiation of deleterious free radical mediated chain reactions in susceptible matrices.

Oxidation of linoleic acid produces hydroperoxide-derived free radicals that attack the chromophore of β-

Table 1 Free and total amino acids composition of CSL after 24 h fermentation

Free amino acid	%	g/100 mL	Total amino acid	%	g/100 g
Ser	6.49	0.1298	Thr	5.437	0.9321
Pro	1.16	0.0232	Ser	2.87	0.4879
Ala	1.09	0.0218	Pro	2.81	0.4777
Cys	1.62	0.0324	Gly	4.198	0.7123
Met	1.65	0.033	Ala	12.13	2.0604
Leu	2.99	0.0598	Val	7.635	1.9271
Tyr	3.77	0.0754	Leu	3.409	0.578
His	4.09	0.0818	Tyr	20.12	3.4238
Lys	77.13	1.5426	Phe	2.1	0.357
			His	9.084	1.5436
			Lys	5.896	1.0013
			Arg	24.31	4.1378

Data presented are the main values (n = 3)

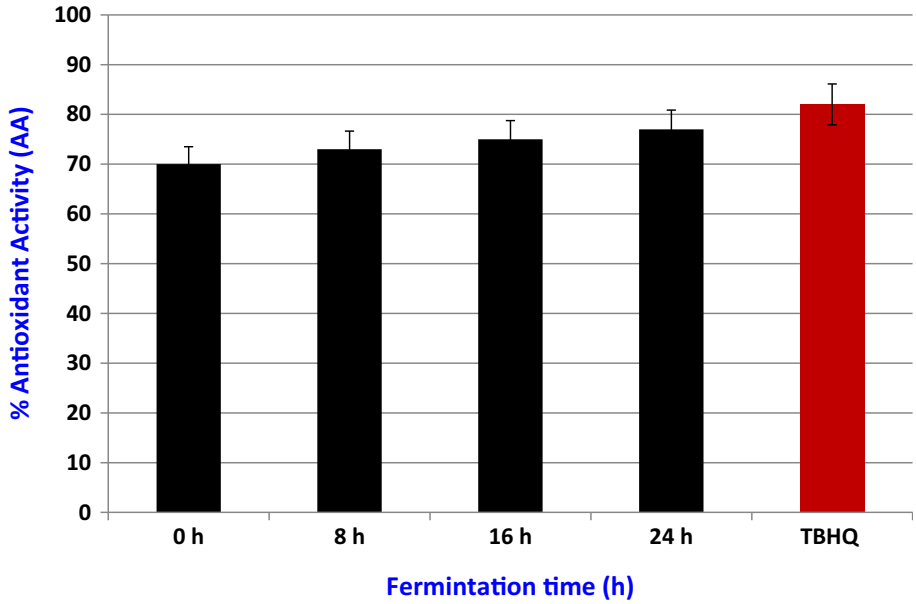

Fig. 1 Scavenging activity of CSL against DPPH· free radical compared with TBHQ. Data presented are the main values (n = 3)

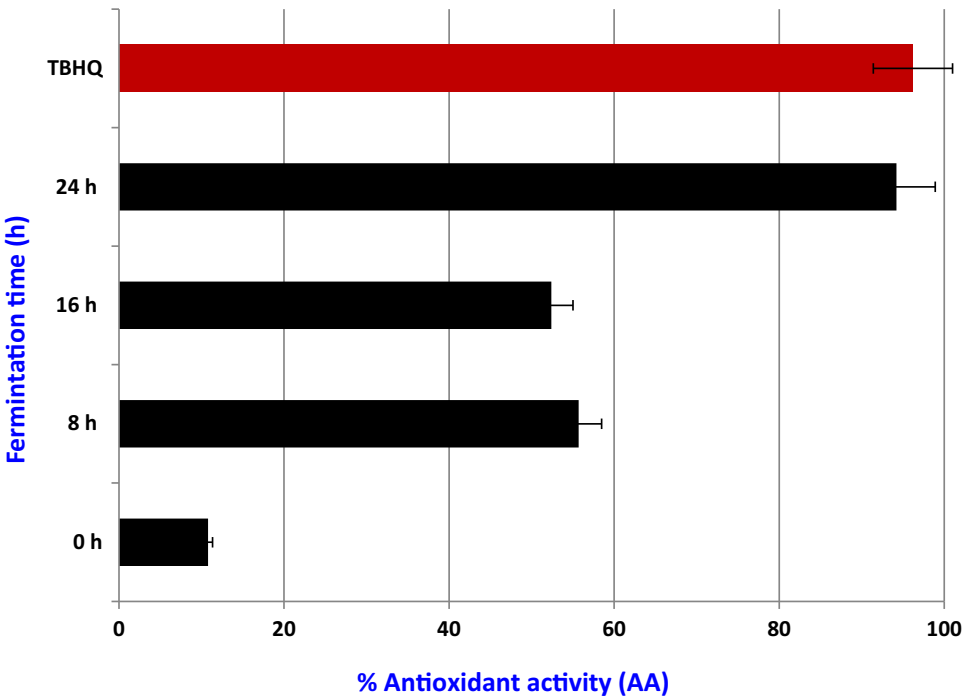

Fig. 2 Antioxidant activity of CSL in β-carotene-linoleic acid system compared with TBHQ. Data presented are the main values (n = 3)

carotene, resulting in bleaching of the reaction emulsion. An extract capable of retarding or inhibiting the oxidation of β-carotene may be described as a free radical scavenger and primary antioxidant [35]. As can be seen in Fig. 2, CSL at different fermentation periods were capable of inhibiting the bleaching of β-carotene by scavenging linoleate-derived free radicals. The results revealed that CSL had comparable scavenging ability to the synthetic antioxidants (TBHQ).

Microorganisms in CSL

Twenty-five isolates were separated from freeze-dried CSL on nutrient broth medium. Fifteen isolates were produced gas in test tubes containing Durham's tube, after incubation for 7 days at 30 °C. Ten isolates were changed the color of the medium to yellow and caused casein hydrolysis.

Data in Table 2 showed that the total count of microorganisms found in CSL after 24 h fermentation. All

Table 2 Total count of microorganisms found in CSL after 24 h fermentation

Test	Result
Total plate count (cfu/g)	1.5×10^3
Total spore formers (cfu/g)	60
Total proteolytics (cfu/g)	7.0×10^2
Total saccharolyticus (cfu/g)	1.2×10^3

Data presented are the main values (n = 3)

the check isolates (identified by Bergeys manual and Biolog technique) formed completely white, round, smooth and shiny colonies. During microscopic examination, all isolates were found to be gram positive long rods. Presence of endospores was confirmed by endospore staining.

Microorganisms are able to grow in a wide range of substrates and can produce remarkable number of products. Changes in the chemical constituents of the fermented liquid can be attributed to bacilli until 8–10 h of fermentation then

Table 3 Identification and characterization of the best isolates in CSL after 24 h fermentation

Characterization	*Bacillus megiterium*	*Bacillus subtilis*
Cultural characterization		
Color	White	White
Size	Large	Medium
Morphological characterization		
Shape	Long rods cause central cell swelling	Long rods do not cause swelling
Mortality	Mobile	Mobile
Endosperm		
Shape	Circle, large	Circle
Location	Center	Center
Stanning		
Gram	G+	G+
Acid fast	Not resistant	Not resistant
endospore	Center, circle, large	Center, circle
Utilization of different carbon source		
No carbon	−	−
Glucose	+	+
Lactose	+	+
Sucrose	+	+
Fructose	+	+
Galactose	+	+
Utilization of different nitrogen source		
Pepton	+	+
Potassium nitrate	+	+
Beef extract	+	+
Yeast extract	+	+
Gelatin	+	+
Casein	±	+
Optimal temperature for growth		
20 °C	−	−
25 °C	−	−
30 °C	+	+
37 °C	−	−
50 °C	−	−
Oxygen requirements		
Aerobic	+	+
Anaerobic	−	−
Facultative	−	−
Microaerophilic	−	−

Ref. [36]

to clostridia until 18 h [15]. *Bacillus subtilis* are important starter cultures for alkaline fermentations observed in traditional fermented legumes [16].

The isolates of *Bacillus* after fermentations at 24 h were assigned according to their biochemical characteristics as shown in Table 3. The ability of sugars and proteins analysis represented in Table 3 according to Bergey [36]. The results were confirmed by identification using Biolog system [31].

It could be concluded that the submerged fermentation of chickpea results in chemical changes of CSL medium. From the analysis of degradation products in the fermentation liquid, over a 24 h period it seems that a considerable substrate modification occurs, as a result of the activity of the growing populations of bacilli, mainly, until about the middle of fermentation. Due to gas formation as an end product of fermentation, CSL might be used as a leavening factor for bakery products. In addition, CSL might be used as an alternative to YE for syngas fermentation because it is rich in nutrients and lower in cost compared to YE.

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