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Bioproduction of beef-like flavour by *Corynbacterium glutamicum* 1220[t] based on enzymatic hydrolyzates of mushroom and soybean supplemented with flavour precursors

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ABSTRACT

Enzymatic hydrolyzates of air dried mushroom and soybean meal were supplemented separately with cysteine and used as main substrates to produce beef-like flavour (M-BF and S-BF, respectively) by *Corynebacterium glutamicum*. Analysis of the free amino acids in the two hydrolyzate samples revealed the presence of 16 amino acids at variable concentrations. During incubation (9 days) the headspace volatiles were isolated by using solid phase microextraction. The results of GC-MS analysis of the isolated headspace volatiles revealed that 2-methyl-3-furanthiol (2-MF) was predominate in both samples after 3 days, whereas after 5 days it was oxidized completely to Bis (2-methyl-3-furyl) disulfide. Sample S-BF comprised the highest yield of 2-MF after seven days whereas M-BF showed the highest content of this compound after 9 days. An opposite trend was found between the biogenerated methanthiol and its oxidation products, dimethyl disulfide and dimethyl trisulfide. A parallel correlation was found between the yield of 2-MF and the odour intensity of each culture during incubation.

Keywords: *Corynbacterium glutamicum*; beef-like flavour; mushroom; soybean meal; methionine.

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INTRODUCTION

The increase in production of processed foods by industrial methods has results in an expanding market for the process flavourings particularly meat-like flavouring. Studies on beef-like flavour have been more widely reported than other meat flavour. Although process flavours may be produced by chemical transformation of natural substances, the resulting products cannot legally be labeled as natural [1]. In this area, consumers generally prefer flavours exhibiting a natural label. Microorganisms play an important role in the generation of natural flavours. However, most of the extensive studies that had been published in this area were dealing with biosynthesis of fruity flavour [2, 3, 4] cheese flavour and coconut aroma [5, 6, 7]. To best of our knowledge, no study was reported concerning the bioproduction of volatiles possessing the characteristic notes of process flavours such as beef-like aroma. However, some studies have been carried out for the biosynthesis of pyrazines and volatile sulfur containing compounds VSCs [8, 9]. Among the great number of volatile compounds present in beef flavour, the sulfur containing compounds, with their low thresholds, make a significant contribution to the distinctive aroma of beef-like flavour [10]. It is well established that VSCs arise primarily from the biodegradation of sulfur/carbon bond of methionine and /or cysteine by the bacteria during fermentation process [11]. Bioconversions of VSCs precursors by lactic acid bacteria (LAB) have been investigated [12, 13]. In LAB, L-methionine degradation could be initiated by a transamination step that leads to 4-methylthio-2-oxobutyric acid (MOBA) which is subsequently converted to methanthiol (MLT) through a chemical process [14,15]

Corynebacterium glutamicum (*C. glutamicum*) is one of the bacteria strains that are believed to be important participants in L-methionine catabolism [16]. *C. glutamicum* is regarded as a promising candidate for biotechnological methionine production to replace current petrochemical based methods that annually supply about 600.00 tons of racemic mixture [17]. Generation of pyrazines from *C. glutamicum* by the addition of acetoin to agar plates at different pH values was studied [9]. The profile of the released volatiles was composed of several pyrazines and acyloin derivatives. Bonnarme et al. [18] compared the capacities to produce volatile sulfur compounds by five yeasts and five bacteria strains. The results revealed the ability of different strains including *C. glutamicum* to generate VSCs from L-methionine such as methanethiol, dimethylsulfide, dimethyl disulfide, dimethyl trisulfide and thioesters. The enzymatic hydrolysis of thioacetate derivatives was found to produce thiols [19, 8,] that seem to impart the characteristic cooked and roasted notes to meat [20].

The effect of cysteine on the ability of some cheese ripening bacteria to produce VSCs from methionine was studied [21]. The highest yield of VSCs was obtained by increasing the cysteine concentration from 0.2 to 1.0 g/l. The results revealed good yield with high reaction rate at pH 5.5-6.5. The ability of lactic acid bacteria to convert cysteine and methionine to VSCs was studied [22]. The presence of methionine degradation products including methanthiol and dimethyldisulfide indicated the possible conversion of cysteine to methionine during the study. A considerable amount of published researches had been focused on the generation of beef-like process flavour by heating hydrolyzed vegetable proteins (HVP) with other flavour precursors via Maillard reaction [23,24]. In current study we used the enzymatic hydrolyzed proteins of each of soybean and mushroom for the production of beef-like flavour by using different thermal technologies [25, 26]. However, to best of our knowledge no study could be found concerning the bioprodcution of beef-like flavour by *C. glutamicum.* Therefore, the main objective of the present study was to evaluate the ability of *C. glutamicum* to produce beef-like flavour from enzymatic hydrolyzate of each of mushroom and soybean with flavour precursor in submerged culture. Comparative data on odour intensity and volatile compounds of the generated beef-like flavours, during fermentation period (9 days), were presented

MATERIALS AND METHODS

Plant materials and chemicals

Defatted soybean meal (48 % protein) was obtained from Food Technology Research Institute, Agric. Res. Center, Giza Egypt and Mushroom (*Agricus bisporus*) was obtained from bloshia mushroom company in Dokki. Cysteine was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Flavourzyme (from *Aspergillus oryzae*) and Alcalase (from *Bacillus Licheniformis*) were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Yeast extract (YE), glucose, peptone, di-ammonium phosphate (DAP) were purchased from Merck (USA). Authentic compounds and standard n-paraffin (C8-C22) were purchased from Sigma Aldrich

Chemical Co. (St. Louis, MO, USA). All others chemicals used in this investigation were of analytical grade and the solvents were purified and distilled before use.

Microorganism, collection and identification

The bacterium *Corynbacterium glutamicum* 1220T, obtained from Microbiological Resources Center (MIRCEN), was cultured and maintained on nutrient agar slant (yeast extract 13 g/l, peptone 10 g/l) at 28°C for 24 h. The direct microscopic method (optical light microscope (10 × 90) Olympus CH40) was carried out for examining the morphological feature of vegetative cells using production medium (enzymatic hydrolyzate of mushroom or soybean meal) during fermentation time as well as Gram staining (Figure1).

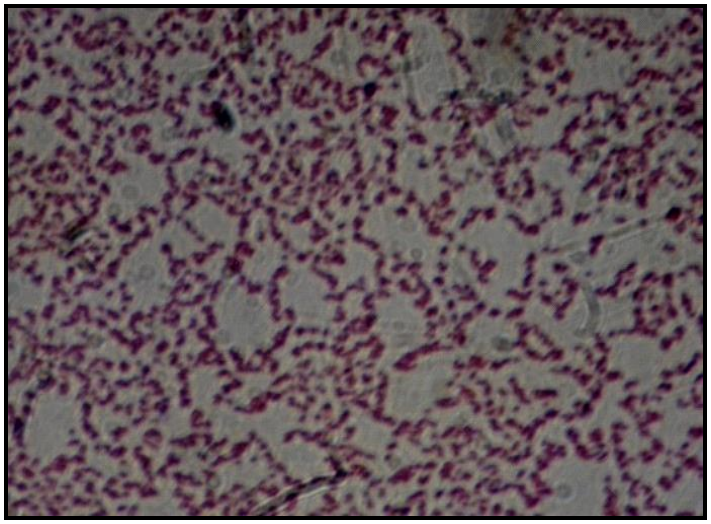

Figure1. Microphotograph of *C. glutamicum* after 3 days culture on production medium (mushroom hydrolysate)

Chemical composition of the substrates

The chemical composition of soybean meal and air dried mushroom were determined as described by AOAC [27].

Production of enzymatic hydrolysate

The enzymatic hydrolyzate of soybean meal or air dried mushroom was prepared according to Aaslyng et al. [28]. Each sample (150 g) was mixed with 825 g of tap water and pasteurized at 85 °C for 5 min. After the mixture cooled to 50 °C, the pH was adjusted to pH 7.0 with 4 M NaOH. Flavourzyme (0.78 g) and Alcalase (0.75 g) were added, and the mixture was allowed to stand without pH adjustment at 50 °C. After 5 h the pH was adjusted to pH 5.0 with 4 M HCl and 14.6 g of NaCl and 0.39 g of Flavourzyme were added. The hydrolysis continued without pH adjustment at 50 °C for a total of 24 h. The enzymes were deactivated at 85 °C for 5 min. After the mixture cooled to 50 °C, the pH was adjusted to pH 6.5 with 4 M NaOH. The prepared hydrolyzates were used as the main substrates for the production of beef-like flavour by *C. glutamicum*. To determine the free amino acids composition, each hydrolyzate was subjected to centrifugation; the precipitate was washed with 300 ml of tap water and centrifuged again. The combined hydrolyzates in water were filtered, freeze-dried (Snijders Scientific b.v. Model L45 Fm-Ro, Tilburg-Holand) and stored immediately in closed glass bottles at -10°C until further analysis. Composition of free amino acids of each hydrolyzate was determined as described in previous study (Lotfy et al., 2015)

Production medium and batch culture of bacterial strain

The *C. glutamicum* was first grown on nutrient broth (YP) medium for 12 h in 250 mL shaking flask with agitation, then inoculated (2%) into the GYP medium which composed of (g/l) glucose 100, yeast extract 10, peptone 30, and di-ammonium phosphate 30 at pH 7.2 and autoclaved at 121^{o}C for 20 min while, glucose

was autoclaved separately. Then GYP medium was inoculated (6%) into production medium which composed of 50 mL sterile hydrolyzate of mushroom or soybean supplemented with sterile cysteine, 2.5 g at pH 5.0 and incubated with shaking (150 rpm) at 28°C for 3,5,7 and 9 days. After that the samples were taken from each treatment for analysis.

Odour sensory analysis

Evaluation of odour quality of the beef-like aroma generated by *C. glutamicum* fermented on the hydrolysates of mushroom and soybean meal was carried out during incubation period 9 days. The evaluation was conducted by a well-trained panel consisting of 10 members (6-female and 4-male) drawn from Food Technology and Nutrition Division, National Research Centre, Cairo, Egypt. All panelists had experience with odour sensory analysis. Furthermore, they were trained for additional 3 h to identify and define the intensity of cooked beef aroma interms of appropriate reference sample. The panelist, sniffed and scored the intensity of the perceived beef-like aroma of each culture medium on the 3rd, 5th, 7th and 9th days on a category scale 0.0(not perceptible) to 10.0 (strongly perceptible). Each sample was evaluated in triplicate.

Isolation of headspace volatiles

Each fermented media was cooled in ice bath and filtrated. The residual was washed with 50 mL distilled water and filtered again. The combined filtrates of each sample with 9.72 µg of 3-heptanol (internal standard) were placed in a 100 mL headspace glass vial sealed with a PTFE faced silicon septum (Supelco, Bellefonte, PA, USA). The volatile compounds in headspace of each sample were extracted by solid phase microextraction (SPME) (Supleco, 57348-U, Bellefonte, PA, USA), with coated fiber of divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS)(coating thickness:50/30µm). The SPME fiber was exposed to the headspace of each sample for 1 h at 65 ºC, then it was inserted into the GC injection port for desorption (260ºC/ 5 min in splitless mode). Before use, the fiber was conditioned in the injection port of the GC (270 ºC/1h) as recommended by manufacture. Extractions were carried out in triplicate.

Determination of reducing and total sugars

Before isolation of the volatile compounds a portion of each filtrate was subjected to analysis of reducing sugar according to DNS method [29] and total sugar according to phenol-H_2SO_4 method [30] using glucose as standard.

Gas chromatography - Mass spectrometry (GC-MS) analysis

A Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett Packard 5970 Mass spectrometer was used for analysis of the volatile compounds. The injection was conducted in the splitless mode for 5 min at 260 ^{0}C. The GC was equipped with a fused silica capillary column DB5 (60 m × 0.32 mm i.d. × 0.25 µm film thickness). The oven temperature was held initially at 50°C for 5 min, and then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate of 1.1 ml/min. The MS was operated in the electron impact mode (EI) (Lofty et al., 2015).). Mass spectra were obtained at 70 eV and scan m/z range from 39 to 400 amu. The retention indices (Kovats index) of the separated volatile components were calculated with reference to the retention time of a series of alkanes (C6- C20) as external standard run at the same conditions. The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology, NIST) and comparison with those of authentic compounds and published data [23,24,31]. The relative areas of the volatile components identified were determined by comparing their peak areas to that of 3-heptanol, an internal standard compound, on total ion chromatograms of GC-MS.

Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) by the Statgraphics package (Statistical Graphics Corporation, 1993; Manugistics Inc., USA). The multiple range least significant difference test (Duncan multiple range test), with significance level at $p< 0.05$, was applied to the results to test the significant difference.

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RESULTS AND DISCUSSION

Chemical composition of substrate

Table (1) shows the chemical composition of the defatted soybean meal and air dried mushroom. The results revealed that protein and lipid content in soybean meal was more twice that in mushroom, whereas, the total sugar showed opposite trend.

Table1. Chemical composition of soybean meal and air dried mushroom

Components	(g/100 g)	
	Soybean meal	Mushroom
Moisture	8.4	7.2
Ash	9.0	10 .0
Lipids	6.0	2.8
Proteins	48.0	17.3
Total sugar	28.6	33.0
Reducing sugar	9.7	0.00

Amino acid content

Table2. Composition of free amino acids of the E-HVP of soybean and air dried mushroom

No	Amino acid	Concentration (mg /100 g)*	
		Soybean meal	Mushroom
1	Aspartic	1.10±0.19	0.32±0.04
2	Threonine	0.56±0.08	1.50±0.20
3	Serin	0.40±0.05	---
4	Glutamic	3.11±0.39	3.84±0.52
5	Alanine	1.43±0.18	3.20±0.43
6	Valine	1.49±0.18	1.53±0.21
7	Methionine	0.29±0.04	0.37±0.05
8	Isoleucine	1.54±0.19	1.42±0.19
9	Leucine	4.21±0.53	2.57±0.53
10	Tyrosine	3.24±0.40	1.31±0.18
11	Phenylalanine	5.74±0.71	1.10±0.15
12	Histidine	3.38±0.42	1.13±0.18
13	Lysine	2.08±0.26	1.77±0.24
14	Arginine	7.47±0.93	0.18±0.02
15	Proline	4.37±0.54	2.16±0.29
16	Aspartic	0.15±0.02	0.63±0.09
Total		40.56±5.46	23.03±3.10

*means of three experiments ±SD, ---: not detected

Table (2) shows the free amino acid contents (mg/100g) of enzymatic hydrolyzed proteins of air dried mushroom and soybean. The results revealed that the soybean and mushroom proteins were principally made up of 16 amino acids with total concentration 23.0 3± 3.10 and 40.56 ± 5.46 mg/100 g (dry weight mushroom and soybean E-HVP, respectively). Glutamic acid was the most abundant free amino acid in enzymatic hydrolyzed mushroom protein (3.84±0.52 mg/100 g) followed by alanine (3.20 ± 0.43 mg/100 g), leucine (2.57±0.53 mg/100 g) and proline (2.16±0.29 mg/100 g). Whereas, phenylalanine was the major free amino acid in the hydrolyzed soybean protein, it comprised (5.73±0.71mg/100 g) followed by leucine (4.21±0.53 mg/100 g), histidine (3.38±0.42 mg/100g), tyrosine (3.24±0.40 mg/100g) and glutamic acid (3.11±0.39 mg/100 g). Methionine was the only sulfur containing amino acid detected in the enzyme hydrolyzed protein of each sample. It was present in low concentration in both samples; however, its concentration in mushroom was higher than that in soybean. The reported amino acids can act as substrates for bioproduction of volatile compounds. A direct biosynthetic link had been demonstrated between generation of pyrazines and the amino acids valine, leucine and isoleucine [9]. Larroche et al. [32] stated that addition of threonine optimize the yield

of some pyrazine derivatives produced by *Bacillus subtillis* IFO 3013 grown in ground soybean suspended in water. Addition effect of different amino acids such as L-proline, ornithine, L-lysine and glutamic acid on the biogeneration of pyrazines and 2-acetyl proline by *Bacillus cereus* was investigated [33]. Biotechnological degradation of methionine gave rise to the production of volatile sulfur compounds (VSCs) such as methanethiol, dimethyldisulfide and dimethyltrisulfide [11].

Culture growth

Table (3) shows the high correlation coefficient between the culture growth (dry matter) and consumed sugar (total and reduced) during fermentation of enzymatic hydrolyzate of soybean (R^2= 0.88 and 0.86, respectively) and mushroom (R^2= 0.92 and 0.87, respectively). Addition of amino acids confirms the fact that *C. glutamincum* grows aerobically on a variety of substrates, such as carbohydrates (e.g. glucose, fructose and sucrose) organic acid (e.g. pyruvate, lactate, citrate and acetate) or alcohol (e.g. methanol) as a single or combined carbon and energy sources [34]. In present study the sugars in the enzymatic hydrolyzate of mushroom and soybean meals act as carbon and energy sources.

Table3. Correlation between sugar content (total sugar and reducing sugar) and culture growth (Dry matter) during incubation time

Incubation time (days)	Soya bean Hydrolysate			Mushroom Hydrolysate		
	Total Sugars	Reducing Sugars	Dry Matter[b]	Total Sugars	Reducing Sugars	Dry Matter[b]
0	50.8[a]	14.6[a]	1.5	53.6[a]	16.2[a]	1.8
3	41.6	13.0	4.6	48.4	14.7	4.9
5	35.5	8.6	5.9	41.3	10.5	5.8
7	31.0	7.3	6.4	36.5	8.6	6.6
9	21.3	5.2	7.1	31.6	6.5	7.9
Correlation coefficient (R^2)	0.88	0.86		0.92	0.87	

[a]Values are g/100 ml fermented culture
[b]Cell dry weight of *C. glutamicum* culture

Volatile compounds

In present study each of enzymatic hydrolyzates of mushroom and soybean supplemented with cysteine was used, as main substrates, to produce beef-like flavour by *C. glutamincum*. The main volatile compounds in headspace of the two *C. glutamincum* cultures were identified, during incubation for 9 days, by matching retention indices, mass spectra with those of reference compounds, if available, or with literature data [23, 24, 31]. The identified compounds included seven volatile sulfur containing compounds (VSCs), three diketones and one pyrazine. It is well documented that the composition of the headspace volatiles depends upon the concentration of the generated volatile compounds in culture media, their vapor pressure and release in the headspace of the culture. So, the detection of the less volatile compounds is difficult. As shown in Table (4) 2-methyl-3-furanthiol (**6**, 2MF), the most important odour impact compound of beef aroma [10] was the major identified compound in the headspace volatiles of both samples after incubation for 3 days. The detected concentration of 3-mercapto-2-pentanone (**7)** in M-BF sample at the being of incubation time (3 days) was approximately twice that in sample S-BF (Table 4). Compounds **6, 7** and 2-ethyl-3, 5-dimethyl pyrazine (**10)** had been generated by fermentation of cysteamine, ethyl-2-lactate and glucose with baker's yeast [35]. The authors concluded that these volatile components, **6, 7** and **10** can be generated by fermentation using suitable precursors and without heating. These odorant compounds have been reported as characteristic constituents of boiled and roasted meat [36]. In present study compound **10** was found in the headspace extracts of the two investigated samples (Table 4). It is one of the pyrazine derivatives biosynthesized by C*. glutamincum* [9]. This compound was reported, among other volatile compounds as impact odorant for roast, burnt note in roasted beef [36, 37].

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Table4. Volatile compounds identified in beef-like flavour (BF) isolated from enzymztic hydrolyzate of mushroom (M-BF) and soybean meal (S-BF) by *C. glutamicum*

No	RT[a]	Volatile components[b]	Relative peak area (%)[c]								ID[d]
			Time of fermentation (days)								
			M-BF				S-BF				
			3	5	7	9	3	5	7	9	
1	<600	Methanthiol	4.03±0.20	5.04±0.25	4.03±0.20	2.60±0.13	3.62±0.18	4.23±0.21	2.67±0.13	2.52±0.13	B
2	608	2,3-Butandione	9.53±0.47	18.04±0.89	14.1±0.70	7.27±0.36	11.06±0.54	10.67±0.53	8.90±0.44	9.9±0.49	A
3	725	2,3-Pentandione	0.28±0.01	0.97±0.05	0.39±0.02	0.36±0.02	0.51±0.03	0.44±0.02	0.52±0.03	0.75±0.04	B
4	740	3-Hydroxy-2-butanone	0.08±0.00	---	0.03±0.00	0.02±0.00	0.24±0.01	---	0.05±0.00	0.06±0.00	B
5	750	Dimethyl disulfide	0.15±0.01	0.17±0.01	0.21±0.01	0.05±0.00	0.12±0.01	0.11±0.01	0.14±0.01	0.12±0.01	B
6	865	2-Methyl-3-furanthiol	19.86±0.99	---	14.37±0.71	22.83±1.13	16.73±0.83	---	20.56±1.02	11.5±0.57	B
7	901	3-Mercapto-2-pentanone	0.11±0.10	---	0.04±0.00	0.03±0.00	0.21±0.01	---	0.07±0.00	0.13±0.01	B
8	924	2-Furanmethanthiol	---	---	0.03±0.00	0.05±0.00	---	---	0.05±0.00	0.22±0.01	A
9	964	Dimethyl trisulfide	0.07±0.00	0.12±0.01	0.11±0.00	0.04±0.00	0.08±0.00	0.20±0.01	0.60±0.03	0.05±0.00	B
10	1075	2-Ethyl-3,5-dimethylpyrazine	0.06±0.00	0.14±0.01	0.17±0.01	0.10±0.00	0.18±0.01	0.17±0.01	0.14±0.01	0.09±0.00	B
11	1537	Bis(methyl-3-furyl)disulfide	---	9.03±0.45	---	---	---	15.00±0.74	---	---	B

[a]Retention indices. [b]Compounds listed according to their elution on DB5 column. [c]Relative percentage of peak area of each compound compared to that of internal standard in GC-MS total ion chromatograms (n=3) ± standard deviation. [d]Volatile compound identification was performed as follows: A: Mass spectrum and retention index were consistent with those of an authentic standard. B: Mass spectrum was identical with that of NIST mass spectrum database, and retention index was consistent with that of the literature (Baek et al., 2001; Song & Xia, 2008; Wu & Cadwallader, 2002). -- Not detected.

Methanthiol (**1)** was identified in both samples, however it's concentration in sample M-BF was higher than that in sample S-BF (Table 4) which may be attributed to the higher concentrate of methionine (0.37±0.05) in E- HVP of mushroom than that in soybean (0.29±0.04). Compound **1** is believed to be produced from one step degradation of L-methionine in presence of L-methionine γ-lyase [38]. It is considered as the precursor of the alkylated sulfides; dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) as well as thioesters [11, 16]. DMDS and DMTS considered as the autoxidation products of methanthiol**.**

L-Methionine degradation can proceed via two degradation steps initiated by aminotransferase that is called also transaminase. This enzyme requires the presence of an amino acceptor such as α-ketoglutrate yielding 4-methylthio-2-oxobutyric acid (KMBA) that is subsequently degraded to methanthiol [15]. The activity of L-methionine aminotransferase was determined in cellular extracts of *C. glutamicum* [18]. Methanthiol and its degradation products DMDS and DMTS as well as hydrogen sulfide H2S were generated by several bacteria from cysteine [22]. The authors suggested that the presence of these products point out to the conversion of cysteine to methionine during the fermentation process.

Three ketones were identified in the present study 2, 3-butandione (**2)**, 2, 3-pentandione (**3)** and 3-hydroxy-2-butanone (**4)** (Table 4). Concentration of compound **4** in S-BF was three fold higher than that in M-BF. This compound is a catabolism product of sugar and commonly observed with *Bacillus subtilis* [39] also was found among the main volatile compounds released by C*. glutamic* used for pyrazines biosynthesis [9] and considered as the precursor of some pyrazine compounds. Compound **2** was the second major compound identified in the two samples. This compound is related to the growth and metabolism of lactic acid bacteria and other microorganisms in several foods [1, 11]

Compound **3** was reported as a degradation product of L-methionine in yeast and bacterium strains [40]. The authors studied the catabolism pathways of methionine in *Geotrichum candidum* and *Brevibacterium lines* and suggested that in both microorganisms methanthiol and α-ketobutyrate were generated. The former compound was considered, as mentioned before, the precursor of other VSCs whereas, the later compound was subsequently degraded to 2, 3-pentandione. This finding is in agreement with that of Ott et al. [41] who suggested that compound **3** was formed from α-ketobutyric acid and active acetaldehyde originating from pyruvate. Then these two precursors can undergo condensation reaction, which is catalyzed by acetate synthase (EC.2.2.1.6), and produce intermediate α-aceto-α-hydroxybutyrate which was subsequently decarboxylated to compound **3**.

As shown in Table (4) the increase in incubation time revealed qualitative and quantitative variations in the biogenerated compounds. The main observation was the absence of compound **6** in the two samples after incubation for 5 days and the generation of its dimer bis (2methyl-3-furyl) disulfide (**11)**. It is known that compound **6** oxidize easily to **11** [26, 42] and both compounds had been identified as the odour impact compounds of beef aroma [10] and other model systems related to meat-like aroma. They give various characteristic aroma described as sweet meat, beef broth and braised beef [43]. In both samples compound **1** showed its highest concentration after fermentation for 5 days followed by gradual decrease during the incubation period 7-9 days. At the same time the oxidative degradation products, DMDS and DMTS, of this compound showed gradual increase in the two samples up to the 7th day followed by a considerable decrease at the end of incubation time (Table 4). The decrease in concentration of methanthiol and DMDS at the end of fermentation time may be attributed to the fact that these two compounds could serve as donors of sulfur and carbon for methionine biosynthesis in C*. glutamicum* [44].

2-Furanmethanthiol (**8)** was detected at low concentration in both samples at 7-9 days. The two thiol compounds **6** and **8** were generated by enzymatic hydrolysis of S-3(3-methyfuryl) thioacetate and S-2-furfuryl thioacetate, respectively in aqueous and organic media using lipase from *Candida rugosa* [8]. These two thiol compounds were shown to be important odorants in commercial meat flavourings as well as cooked beef and chicken broth [45]. Compound **8**, which possesses roast coffee aroma, was identified in the volatiles of fermented beverages [11] This compound can be produced by interaction of the sugar degradation products, which released during sterilization of sugar (121°C), and hydrogen sulfide, catabolism product of cysteine [22].

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Odour sensory evaluation

The changes in the intensities of the beef-like flavour (M-BF and S-BF) of the two fermented cultures (hydrolyzate of mushroom and soybean meal, respectively) during incubation for 9 days were followed. The odour intensity was scored by 10 panelists. Three replicates were applied to assess the results (Figure 2). After 3 days both cultures showed considerable high odour intensity. However, M-BF showed higher score than S-BF. After 5 days both samples showed dramatic decrease which may be attributed to the absence of 2-methyl-3-furanthiol(Table 4), the odour impact compound of beef aroma [10, 46]. The odour intensities of both samples showed significant ($P< 0.05$) increase after 7 days; however sample S-BF showed the best results followed by a significant decrease at the end of fermentation time (9 days), whereas, sample M-BF showed the best results after 9 days. These results confirm those of GC-MS analysis of the headspace volatiles of each fermented sample (Table 4). There was a quite agreement between the odour intensity and the yield of 2-methyl-3-furanthiol of each investigated sample.

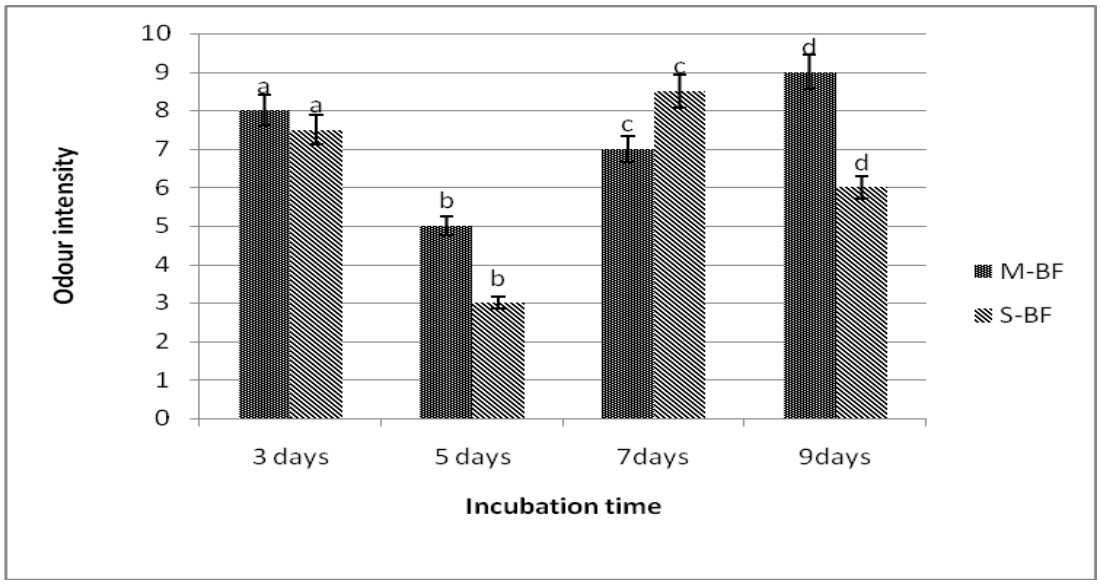

Figure2. Odour intensity of beef-like flavour (M-BF and S-BF) generated during fermentation of the hydrolyzates of mushroom and soybean meal, respectively, by *C. glutamicum.*

CONCLUSION

Addition of the appropriate precursors to the culture medium promotes the biosynthesis of the desired flavour. Enzymatic hydrolyzates of wild mushroom (*Agricus bisporus)* and soybean meal supplemented with cysteine can be separately used as the main substrates for the biogeneration of beef-like flavour by *C. glutamicum.* The results of the present study confirm the fact that sulfur containing precursors is necessary for biogeneration of beef-like flavour.

In previous studies addition of different amino acids, free of sulfur, optimized the bioproduction of nitrogen containing compounds such as pyrazines. However, in present study the presence of methionine and cysteine in the culture media of *C. glutamicum* gave rise to high production of volatile sulfur containing compound, in particular the thiol containing compounds such as 2-methyl-3-furanthiol which considered as a potent odorant of beef-like flavour. At the same time the nitrogen containing compounds such as pyrazines showed weak representation. A quite agreement was found between the odour intensity of beef-like flavour and the yield of 2-methy-3-furanthiol during incubation of the cultures.

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