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Production of single cell protein from Stickwater of kilka fish meal factory using *Lactobacillus plantarum* and *Bacillus licheniformis*

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Abstract: One of the most important problems in kilka meal factories is stick water production, as a waste, from meal production process. Five hundred litters of stick water are produced for each 1000 kg of kilka fish. This waste has rather high protein and might be used as substrate for the growth of bacteria. In this study, *Lactobacillus plantarum* and *Bacillus licheniformis* were used for SCP production from stick water of kilka fish factory. Adaptation of *L. plantarum* and *B. licheniformis* to pure stick water was performed by increasing the concentration of stick water in distilled water. Treatments included control (lake of stichwater), 50% stichwater and 100% stichwater. The growth of these bacteria was studied by spectrophotometric method and reading the optical density of bacteria at 600 nm. After separation of biomass, the final product was analyzed for amount of biomass, amino acid profiles, total protein, moisture and ash. The highest amount of biomass production using *L. plantarum* in the control treatment, 50% and 100% stickwater was 3.56, 4.55 and 5.75 g/L, respectively. Biomass production using *B. licheniformis* was also 2.43, 3.67 and 4.78 g/L, respectively. The results showed that amino acids composition in produced SCP is comparable with the suggested pattern of requirement by FAO/WHO and NRC. Based on to the results, application of pure stickwater was suitable for production of *L. plantarum* and *B. licheniformis*.

Key words: *Lactobacillus plantarum; Bacillus licheniformis; SCP; Stick Water; Kilka fish meal*

1. Introduction

Single Cell Protein (SCP) at the first time was introduced by Carl Wilson in 1944. SCP in terms called cells or proteins derived from microorganisms, that following the growth of microorganisms on different sources of protein and carbohydrates was produced and used as an alternative or supplementary source of food in the diet of animals and humans (Jamal et al., 2007; Adedayo et al., 2011). Various microorganisms such as bacteria, yeast, algae can be used for SCP production (Kurbanoglu and Algur, 2002). Due to the rapid growth of bacteria, the quality and amount of protein (up 70%), bacteria had a significant advantage over other microorganisms for SCP production (Anupama and Ravindra, 2000). Among the most important bacteria that used for the production of SCP, it can be noted the various species of Pseudomonas, Bacillus and lactic acid bacteria (Erdman et al., 1997). In most cases, SCP was produced from waste of feed industries and few studies related to SCP production from waste and residues aquatic done. Aquatic organisms due to their high protein and essential fatty acids have been extremely valuable and these wastes can be used as a substrate for the SCP production (Lunar et al., 2006).

One of the most important problems in kilka meal factories is stick water production, as a waste, from meal production process. Five hundred litters of stick water are produced for each 1000 kg of kilka fish. Unfortunately, these materials have been released directly to the environment and because of the high fat and protein and subsequent activation of proteolytic and lipolytic bacteria caused additional pollution in the environment (Kam et al., 2012). This waste has rather high protein and might be used as substrate for the growth of bacteria such as LAB and bacillus.

In the present study, we investigated production of single cell protein from stickwater of kilka fish meal factory as medium using *L. plantarum* and *B. licheniformis*.

2. Material and methods

2.1. Microorganisms and mediums

B. licheniformis (PTCC 1355) and *Lactobacillus plantarum* (PTCC 1058), was obtained from the Persian Type Culture Collection which was prepared as a lyophilized stock. Lyophilized stocks of these bacteria was cultivated in TSB (Tryptic Soy broth) (Merck, Germany) and MRS (De Man Rogosa & Sharp broth) (Merck, Germany) and incubated at 35˚C for 24 h, then centrifuged in 5000 rpm for 5 minute.

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2.2. Stickwater

In this study, the stickwater was obtained from the kika fish meal factory (Shiner factory, Behshar, Mazandaran, Iran) and kept at -20◦C for future study. The stickwater was filtered by Whatman filter paper 1 and autoclaved for 15 min at 121◦C.

2.3. The characteristics of the stickwater

The characteristics of different treatments of stickwater were determined according to procedures described in the standard methods (American Public Health Association (APHA, 1998). The elemental of stickwater were measured using a photometer (Hitachi, Japan) with standard method described by APHA (1998). The crude lipid of stickwater was estimated according to the method described by Bligh and Dyer (1959). The percentage amounts of dry weight, moisture, and ash were estimated by AOAC methods (AOAC, 2003). Also, the chemical oxygen demand and biochemical oxygen demand (BOD) of stickwater were determined according standard method described by APHA (1998).The crude protein content was expressed as total nitrogen multiplied by 6.25. The total nitrogen was determined by Kjeldahl method (Kjeltec Analyzer Unit 2300).

2.4. Batch culture

For SCP production, 10% from 0.5 McFarland (1.5×10^{8}) were taken for *B. licheniformis* and *Lactobacillus plantarum* respectively, containing the standard medium and stickwater. The incubation time for maximum biomass generation was determined for *B. licheniformis* and *Lactobacillus plantarum* by and found to be 120 h for control treatment (standard medium) and stickwater treatments. The microorganism was grown for 5 days (the end of the exponential phase). The cell concentration of the cultured media of *B. licheniformis* and *Lactobacillus plantarum* was determined by the cell optical density at 600 nm with a spectrophotometer (Hitachi, Japan). The biomass was also measured, based on the standard calibration curve of absorbance as a function of the biomass for pure cultured *B. licheniformis* and *Lactobacillus plantarum*. The biomass *B. licheniformis* and *Lactobacillus plantarum* was harvested by filtering the cultured medium through filter paper (Whatman 42). After harvesting, the biomass was washed with de-ionized water followed by drying at 105 ◦C until constant weight was attained (Zhang et al., 2008). The amount the protein, moisture and ash percentage in *B. licheniformis* and *Lactobacillus plantarum* at the peak of growth in the control and stickwater treatments (50% and 100% stickwater) was determined. The amino acid profiles at maximum growth time were measured in the control treatment and compared with stickwater treatment. For the amino acid measurement, the *B. licheniformis* and *Lactobacillus plantarum* samples were centrifuged for 10-15 min at 5000 rpm, and then washed with de-ionized water to remove soluble, residual nitrogen salts. The protein, moisture and ash percentage and amino acid profiles were duplicated.

2.5. Amino acids analysis

The amino acids analysis was carried out by the Pico-Tag technique that consists of three steps: (a) Hydrolysis of protein or peptide samples to yield free amino acids, (b) pre-column derivatization of the samples with PITC, and (c) analysis by reverse phase HPLC (SUPELCOSIL, LC-DABS, and USA). The chromatographic separation on the hydrolyzates was performed using a reverse phase Pico-Tag column (4.6×120 mm) C18 at 38 ◦C and a UV detector Vis at 436 nm (Ovissipour et al., 2009). The chemical score of the SCP was computed according to Kam et al., (2012), relative to the essential amino acid (EAA) profile in a standard protein as described by FAO/WHO (1990) and NRC (1993) was compare to our results.

3. Results

The characteristics of the stickwater were summarized in Table 1. The highest amount of biomass production using *L. plantarum* in the control treatment, 50% and 100% stickwater was 3.56, 4.55 and 5.75 g/L, respectively. Biomass production using *B. licheniformis* was also 2.43, 3.67 and 4.78 g/L, respectively. The amount the protein, moisture and ash percentage in *B. licheniformis* and *Lactobacillus plantarum* at the peak of growth in the control and stickwater treatments (50% and 100% stickwater) was showed in Table 2 and Table 3. The quality and quantity of amino acids profiles in bacteria Single Cell Protein produced stick water in Kilka fish meal proceesing was showed in Table 4 and compare to the suggested patterns of requirement by FAO/WHO and NRC.

Table 1: Characteristics of stickwater

Parameter	value*
Potassium (mg/l)	1454±126
Calcium (mg/L)	2155±156
Na (mg/L)	110±8.6
Nitrate (mg/L)	231±10.8
Nitrite (mg/L)	0.65±0.003
Ammonium (mg/L)	0.16±0.001
COD (mg/L)	6300±586.3
BOD (mg/L)	2520±185.4
Total lipid (mg/100 g dry sample)	0.075±0.004
Protein (percentage in 100 g dry sample)	68.25±1.04
pH	6.45±0.6
TS (mg/L)	4.5±0.06
TSS (mg/L)	0.047±0.001
TDS (mg/L)	4.32±0.02
TVA (mg/L)	0.311±0.001
VSS (mg/L)	0.075±0.002

* Values are the means ± standard deviation of three determinations

Table 2: Quantitative analysis of dry matter produced from *Lactobacillus plantarum* in different treatments of stick water

Protein %	Ash%	Dry mater%	Moisture%	Treatment
0.29^{b} ±68.29	0.029^{c} ±3.29	0.136^{c} ±93.29	0.12^{a}* ±3.56	Control
0.26^{a} ±69.71	0.11^{b} ±4.17	0.26^{b} ±97.71	0.02^{b} ±2.11	stick water 50%
0.35^{a} ±70.21	0.15^{a} ±5.26	0.31^{a} ±98.11	0.12^{c} ±1.12	stick water 100%

*Means with different letters in a row differ significantly (P<0.05)

Table 3: Quantitative analysis of dry matter produced from *Bacillus licheniformis* in different treatments of stick water

Protein %	Ash%	Dry mater%	Moisture%	Treatment
0.39^{a} ±70.17	0.026^{b} ±3.45	0.171^{c} ±94.37	0.016^{a}* ±3.11	Control
0.17^{a} ±69.36	0.071^{a} ±4.49	0.065^{b} ±95.26	0.036^{b} ±2.78	stick water 50%
0.32^{b} ±68.91	0.09^{a} ±4.95	0.051^{a} ±97.30	0.013^{c} ±2.29	stick water 100%

*Means with different letters in a row differ significantly (P<0.05)

Table 4: Quality and quantity of amino acids profiles in bacteria and fungal Single Cell Protein produced stick water in Kilka meal processing plants

Amino acids	Lactobacillus plantarum (g/ 100 g^{-1})	Bacillus licheniformis (g/ 100 g^{-1})	Fish meal (g/ 100 g^{-1})	Reference Protein 1^{a}	Reference Protein 2^{b}
Aspartic acid	7.83	4.73	8.60	-	-
Glutamic acid	9.43	6.36	13.4	-	-
Serin	2.81	3.79	4.10	-	-
Glycine	3.14	3.50	9.30	-	-
Threonine	3.71	2.74	3.8	0.9	3.9
Histidine	3.62	1.49	2	1.6	2.1
Alanine	8.75	3.50	6.3	-	-
Proline	3.15	4.17	5.5	-	-
Arginine	3.53	2.67	6.1	-	1.31
Tyrosine	2.74	3.11	2.80	-	-
Valine	4.65	2.88	4.5	1.3	3.6
Methionine	1.37	1.45	2.40	1.7	3.1
Isoleucine	3.62	2.17	3.8	1.3	2.5
Leucine	4.36	2.34	6.4	1.9	3.3
Phenylalanine	2.81	2.25	3.4	-	6.5
Lysine	4.13	4.67	6.7	-	-
Cysteine	-	-	0.9	-	-
AAΣ	34.54	24.19	37.99	-	-
NAAΣ	35.11	25.61	50	-	-
NAA/Σ AAΣ	0.98	0.94	0.75	-	-

RP_1: Chemical score calculated with FAO/WHO reference protein as the base.
RP_2: Chemical score calculated with amino acid requirements as per NRC (requirements of common carp)

4. Discussion

In this research, biomass production was one of the parameters used to survey the potential of microorganism production from the SCP. The stickwater wastewater was treated batchwise with suspended cells of *L. plantarum* and B. licheniformis and compared with standard medium as control. The temperature and the pH of growth medium were optimized for each bacteria. The fermentation was conducted for 5 days. The highest amount of biomass production using *L. plantarum* in the control treatment, 50% and 100% stickwater was 3.56, 4.55 and 5.75 g/L, respectively. Biomass production using *B. licheniformis* was also 2.43, 3.67 and 4.78 g/L, respectively Compared to the results of the *L. plantarum*, the biomass concentration was higher for *B. licheniformis* grown on stickwater. Stickwater had a significant effect on the biomass production reduction for both *L. plantarum* and *B. licheniformis*. These bacteria are more active when grown on the medium containing stickwater as carbon source for synthesis of single cell protein. Stickwater wastewater contains different compounds (mineral matter, lipid, and protein) which may be used as carbon and energy sources for the growth of the bacteria in the production of single cell protein.

Maximum bacterial biomass production occurs nearly in end of fermentation period. The diminution in biomass may be due to either the exhaustion of the carbon source or the cell autolysis, which is in agreement with the findings in other studies regarding single cell protein production by microorganisms (Zhang et al., 2008). Other authors have investigated many bacterial biomasses grown in different substrates (Jamal et al., 2007; Konlani et al., 1996; Kurbanoglu and Algur, 2002; Lee and Kyun Kim, 2001; Mahat and MacRae, 1992; Nigam, 1998, 2000; Schultz et al., 2006; Zhang et al., 2008).

The maximum amount of biomass production of 5.75 g/L in this study was higher than the average reported for *Candida utilis* (5.1 g/L), and *Trichoderma viride* WEBL0702 (5.54 g/L) grown on molasses, and winery wastewater treatment, respectively (Nigam, 2000; Zhang et al., 2008).

However, the maximum amount of biomass production was 11.48 g/L in *Mucor hiemalis* in presence of wheat flour and *Candida utilis* (7 g/L) grown from pineapple cannery effluent (Lee and Kyun Kim, 2001; Jamal et al., 2007). The reason for the difference between other investigators and this research might be due to the type of bacterium and fungus or used mediums (Konlani et al., 1996; Mahat and MacRae, 1992; Nigam, 2000).

4.1. Proximate factors of SCP

Tables 2 and 3 exhibit the amount of dry matter, moisture, ash and protein in the biomass of *L. plantarum* and *B.licheniformis* with control treatment and stickwater when biomass had reached peak values. The amount of dry matter, moisture, and ash made a significant difference in the control treatment compared to stickwater treatment.

The results of this study showed that *L. plantarum* and *B.licheniformis* on stickwater (50% and 100%) had similar percent of dry matter compared to control treatment. Percentage of ash in biomass produced by *L. plantarum* was higher than that of the biomass produced by *B.licheniformis*. Percentage of protein in the biomass produced by *L. plantarum* on stickwater treatment was a little higher than in the biomass produced in the control treatment, but this value in *B .licheniformis* was lower than compared to control treatment.

In general, the protein content in production of the single cell for protein should be between 39 to 73% (Gao et al., 2007; Patil et al., 2000). Many fungal species are used as protein rich food (Anupama, 2000; Frazier and Westhoff, 1990; Zhang et al., 2008). The production of fungal biomass protein produced by *A. niger* was investigated with the treatment of winery wastewater (Zhang et al., 2008). The authors reported that SCP produced by *A. niger* was 36.6%. The corresponding biomass production and COD reduction were 5.50 g/L and 87.0%, respectively. These comparative results are important, since the process for SCP production will be environmentally and economically sustainable if biomass is produced on stickwater wastewater.

The composition of amino acids in the biomass of *L. plantarum* and *B.licheniformis* is shown in Table 4. The results of this study indicate that the SCP produced by *L. plantarum* and *B.licheniformis* has all the essential amino acids. The composition of the EEA of *L. acidophilus* and *A. niger* appears comparable to protein in the FAO standard and in SCP from other sources (Anupama, 2000; Erdman et al., 1977). The aspartic, glutamic, alanine, Leucine, valine and lysine produced by *L. plantarum* biomass and aspartic, glutamic, lysine and proline in *B. licheniformis* biomass were higher than other amino acids. The maximum and minimum amount of amino acids produced by *L. plantarum* biomass in the stickwater (100%) were glutamic acid (9.43%) and methionine (1.37%).The maximum and minimum amounts of amino acids, glutamic acid (6.36%), and methionine (1.43%) produced by *L.licheniformis*. Among nonessential amino acids (NEAA) in *L. plantarum* and *L.licheniformis* biomass, aspartate and glutamate were in the highest concentration. The EAA profiles of the SCP produced by the *L. plantarum* and *L.licheniformis* biomass are in agreement with FAO reference protein and are comparable with some other food proteins such as fish meal, carp and FAO/WHO standard. Methionine is known to be the limiting amino acid in SCP (Fabregas and Herrero, 1985; Kim and Lee, 2000; Shipman et al., 1975). Our results show that *L. plantarum* biomass clearly had almost the same quantity of methionine as called for by the FAO/WHO reference protein. Methionine is somewhat labile toward acid hydrolysis. Therefore, the values presented in Table 4 for methionine underestimated the actual methionine concentration in the SCP of the *L. plantarum* and *B. licheniformis* (Fabregas and Herrero, 1985; Menden and Cremer, 1970). The amino acids composition of *B. licheniformis* according to FAO/WHO standard is well balanced (Anupama, 2000; Kuzmanova et al., 1989). The essential amino acids such as leucine, methionine, and lysine in SCP are very important for the growth of marine fish. For example, lysine is reported to be able to stimulate the growth of marine animals (Gao et al., 2007; Stottrup and McEvoy, 2003).

Many fish require high levels of good protein in their diets, but the use of dietary protein for growth depends ultimately upon the availability of a suitable balance of amino acids (Barroso et al., 1999; Kim and Lee, 2000). The amount of crude protein of *L. plantarum* and *B. licheniformis* biomass grown on stickwater wastewater in this study is comparable to and the highest among the amounts in many corresponding microorganisms reported in the literature. The amount of crude protein produced by *Rhodopseudomonas palustris* was 72–74%, and the amino acid profile of the protein compared favorably with the FAO guideline (Kim and Lee, 2000). *Cellulomonas biazotea* cultivated in perennial grass produced biomass with 60% protein and 10% RNA, and the biomass also contained all desired amino acids, with isoleucine as the limiting acid (Rajoka et al., 2006). *Kluyveromyces marxianus* CBS65560 bacterium grown on deproteinized whey concentrates as medium showed a distinct increase of 8 out of 10 EAA compared to sweet and sour whey protein, exceeding the World Health Organization (WHO) guidelines for valine, leucine, isoleucine, threonine, phenylalanine, and tyrosine (Schultz et al., 2006). *Methylomonas* sp. cultivated on natural gas as medium produced biomass with 69.3% dry weight protein (Yazdian et al., 2005). *Rhodocyclus gelatinosus* cultivated on a poultry slaughterhouse wastewater medium produced the following composition: 67.6% crude protein and 4.2% ash of biomass (Ponsano et al., 2003). The amount of crude protein in *Candida utilis* NRRL-Y 900 grown on pineapple cannery effluent were 55.3 dry weights. The yeast had a balanced amino acid profile, except for sulphur-containing amino acids (Nigam, 1998). In

other studies, the amount of protein attained by *Trichoderma viride* was 31.9% with lemon pulps as medium (De Gregorio et al., 2002), by *Candida krusei* was 47–50% with sorghoum hydrolysate as medium (Konlani et al., 1996), by *Scytalidium acidophilum* was 44–47% grown on acid hydrolysates of waste paper (Ivarson and Morita, 1982), and by *Rhizopus oligosporus*on was 50.2% grown on natural rubber waste serum (Mahat and MacRae, 1992). *Schwanniomyces castellii* B5285 grown on cassava starch as medium produced 45.6% protein with methionine as the limiting amino acid (Hongpattarakere and H-Kittikun, 1995). *Candida langeronii* cultivated on sugar cane bagasse hemicelluloses hydrolysate produced biomass with 48.2% dry weight protein and 5.8% dry weight RNA (Nigam, 2000).

5. Conclusion

The results of the present study showed that of *L. plantarum* and *B. licheniformis* can be used successfully for the production of SCP with stickwater as substrate. A maximum of 5.75 g/L of *L. plantarum* biomass was produced with stickwater as substrate, while the biomass produced by *B. licheniformis* contained 4.78 g/L. In summary, it may be feasible to develop a biotechnological treatment process for use of stickwater wastewater with high COD reduction and high biomass production, producing SCP using stickwater wastewater as a low-cost substrate.

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