Production of single cell protein from natural gas: Parameter optimization and RNA evaluation

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Abstract

Production of single cell protein (SCP) from natural gas in a one liter bubble column reactor and optimization of the process parameters were investigated. The medium specifications, nitrogen sources, initial inoculum volume, and inlet ratio of gas to air were considered as process parameters to be optimized. The optimum condition for highest biomass production in which the maximum quantity of protein was obtained, were the use of a certain carbon-less salt broth utilizable with methane (named as Methane Salt Broth/MSB) and sodium nitrate as medium and nitrogen source respectively. Also, 7%(v/v) inoculation size, and an inlet gas mixture of 60/40 natural gas/air were determined as the effective inoculation and appropriate volume configuration of inlet gases. Protein production in optimum condition was 69.3%(w/w) of biomass in dry basis which its structural amino acids can be in comparable with other nutrient sources. An average amount of 10 g RNA out of 100 g of cellular protein [or 6%(w/w) RNA in whole biomass] was extracted from the biomass which is extremely near to the possible minimum of RNA distribution among bacteria. Heat shock treatment was applied for reducing the RNA in the biomass bulk. Heat Treatment at 60 to 65°C for 10 to 20 min provided the best RNA reduction results (around 1 gram in 100 grams of protein). Regarding the structural amino acids and RNA content, the properties of single cell protein resulted in this experimental work, were in a frame which it could be consumed safely.
Keywords: *Single Cell Protein (SCP); Natural Gas, Methylomonas; Amino Acids; Taguchi Method; RNA Measurement*

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INTRODUCTION

Despite of low solubility, flammability, and non-high-purity in natural sources, methane is a good candidate for single cell protein (SCP) production for its non-toxicity, selectivity, and volatility (Anthony, 1982; Tani, 1985; Hetland, 1998). Therefore, several investigators have shown the ability of methane for being utilized by relevant organisms for protein production from cheap and vast carbon sources; i.e. natural gas (Woolard and Eastman, 1973; Moribaga *et al.*, 1976; Faust and Prave 1983; Berge *et al.*, 2005; Schøyen *et al.*, 2005). Hence, natural gas as a carbon source could be placed among other potential substrates which results in saving world from protein scarcity (Shojaosadati *et al.*, 1996; Faraidouni and Shojaosadati, 1998; Shojaosadati *et al.*, 1999; Anupama and Ravindra, 2000).

Since methane has formed the largest part of natural gas [85 to 90 (v/v)%] and Iran owns second largest natural gas resource in the world, SCP production from this substrate could be economically reasonable due to accessibility and cheapness of natural gas resources. This subject, however, in view point of increasing the rate of hygienic food consumption and availability of huge natural gas in Iran, has never been considered technically.

Therefore, first isolation of potential microorganisms and suitable growth conditions has to be investigated experimentally. In this study, variables including: growth mineral medium, nitrogen source, inoculum volume, and concentration of methane in inlet gas

as challengeable parameters affecting optimal bacterial growth were selected. Since, RNA content of a bacterial-source protein, has to be reduced and controlled in downstream processing (for example for human use), an RNA concentration test during heat shock processes were investigated (Chomczynski, 1993, Shojaosadati *et al.*, 1999).

MATERIALS AND METHODS

Collection, enrichment, and purification of microorganisms: For collecting appropriate organisms capable of utilizing natural gas into biomass, 25 liquid and 25 solid samples were taken from rivers and soils of an oil-rich area of *Masjed Suleyman*, in southern part of Iran. Samples were stored in sterilized tubes at 4°C before experiments. Sealed flasks (each 500 ml), were used for initial cultivation experiments. Flasks were exposed under a vacuum level of –720 mmHg for 24 h for leakage test in body and connections. Two grams of each sample was added to 100 ml of a certain carbon-less salt broth utilizable with methane (named as Methane Salt Broth/MSB; MSB specifications will be given in the section of "growth media and their preparation") in a sealed flask and incubated at 32°C and 200 rpm in a shaking incubator for 7 days. Each flask initially contained 30% (v/v) air and 70% (v/v) natural gas.

Second stage of enrichment was performed by addition of 10 ml of each incubated sample to the same amount of the MSB medium with the same incubation condition as before. 1 ml of each culture was diluted by a factor of 1000 and 0.1 ml added to the Petri plates containing Methane Salt Agar (MSA). MSA was prepared by adding 15 g/l agar-agar to the MSB medium.

For generation of the microbial bulks in the form of colonies, the plates were transferred to a tapped desiccators which was gassed by a mixture of natural gas and air [70 (v/v)% and 30 (v/v)% respectively], twice a day each time for 1 min. The container was kept in this condition for 14 days at 32°C. For purification and having unique colony forming culture, one loop of the biggest and most uniform colony was picked up and inoculated on another plate and cultivated by the same method. This procedure was repeated for more than 3 times until same colonies in color, size, shape, and density were received. This, however needed to be repeated 3 to 5 times. Among 18 colonies with satisfactory growth in the defined condition, one with the most desired appearance in size and uniformity was selected for inoculum preparation. The inoculum was generated by a loop of the selected colony incubated in

a 100 ml MSB with the same conditions as described before.

Growth media and their preparation: The MSB medium contained (g/l): 0.020 $CaCl_2.2H_2O$; 0.010 $FeSO_4.7H_2O$; 1.400 KH_2PO_4; 0.600 $MgSO_4.7H_2O$; 0.050 NaCl; 2.200 NaH_2PO_4; and 3.000 $(NH_4)_2SO_4$. For trace elements 0.5 ml from the following solution was added to the mixture as trace elements (g/l): 4.000 $CoCl_2.6H_2O$; 0.050 H_3BO_3; 12.100 $MnSO_4.4H_2O$; 1.530 $NaMoO_4.2H_2O$; and 3.200 $ZnSO_4.7H_2O$. In order to avoid undesired reaction between chemical compounds during sterilization process, i.e. 121°C and 1140 mmHg, each salt solution was autoclaved individually for 15 min. After cooling down to the room temperature, they were mixed together in a complete sterilized condition. Due to the chemical decomposition, $FeSO_4.7H_2O$ was sterilized using a micro filter (0.2 μm). Finally, the pH of medium was set in the range of (6.8-7.0) using 1N sterile HCl or NaOH. Methane Salt Agar (MSA) which was the medium used for purification of microorganism on Petri plates was prepared by adding 15 g/l agar-agar in the MSB medium.

Two other media were prepared for testing in the bubble column: Nutrient Mineral Salts (NMS, Svenning *et al.*, 2003) and a lab-synthesized Medium without Carbon (MWC, Morinaga *et al.*, 1976). NMS contained (g/l) 0.025 $Ca(NO_3).7H_2O$; 0.004 $CuSO_4.5H_2O$; 0.014 $FeSO_4.7H_2O$; 1.600 KH_2PO_4; 0.080 $MgSO_4.7H_2O$; 1.180 $NaNO_3$; 1.160 Na_2HPO_4; and 0.034 $ZnSO_4.7H_2O$; whereas MWC contained (g/l) 0.001 $CuSO_4.5H_2O$; 0.010 $FeSO_4.7H_2O$; 0.300 KH_2PO_4; 0.200 $MgSO_4.7H_2O$; 1.800 Na_2HPO_4. $12H_2O$ and 0.500 $(NH_4)_2SO_4$.

Preliminary identification of microorganisms: Biochemical and physical tests (include: oxidase, catalase, Gram strains, cell shape, motility, and color) were performed on the basis of Bergey's Manual (Boone and Castenholz, 2001). The characteristics of the microorganism were: single rod or branched rod (but not helical), motile, Gram–negative, and aerobic (temperature range for growth was 28 to 35°C). The isolated cells demonstrated a desired growth on methane, methanol, and formaldehyde which is in accordance to the results given in Bergey's manual (Boone and Castenholz, 2001). Specific approaches were done according to the guide-method given by Murrell and McDonald (Murrell and McDonald, 2000). MWC medium, supplemented with DMSO (dimethylsulfoxinate), methylchloride, and formic acid (as carbon suppliers), inoculated by isolated cells. Based on the

results of these experiments, isolated microorganisms were distinguished as strains belong to *Methylomonas* family.

The bubble column reactor: As a previous practice (Hosseini *et al.*, 2003), a one liter batch glass made
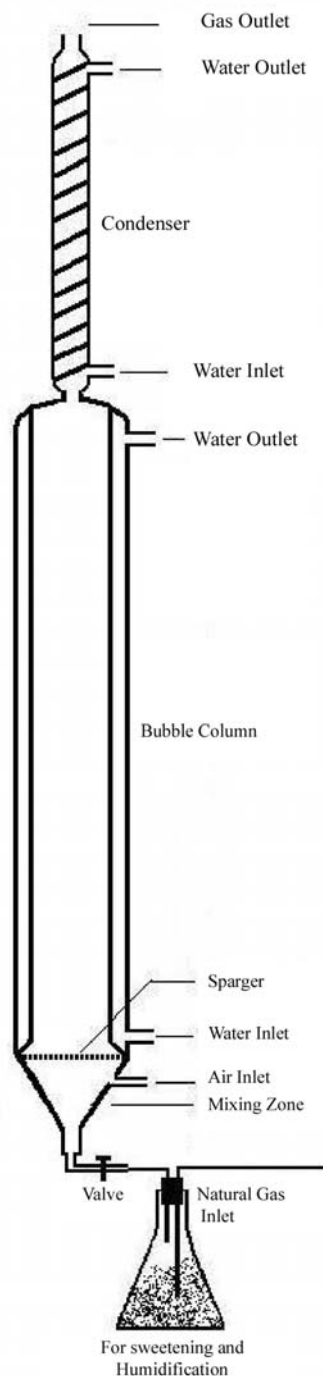

Figure 1. A schematic representation of the bench scaled bubble column bioreactor for production of single cell protein from natural gas.

column (51.0 cm in height and 5.0 cm in internal diameter), as Figure 1 shows, was selected for biomass production in larger scale. Natural gas and air streams (with different flow rates) were entered through separate lines and mixed at the bottom of the reactor and sparged inside through 40 fine holes having diameters of 0.25 cm. To prevent evaporation a condenser was installed on the top of the column. For all experiments, reactor temperature and pH were adjusted at 32°C and 6.8-7.2 by a heat controllable water bath and 1.0 N HCl/NaOH respectively, and 750 ml of medium containing inorganic compounds added to the column. The biomass growth was calculated through changing the broth turbidity measured by a UV visible spectro-photometer (Cary 50 Conc., Spain) calibrated at 600 nm.

Optimization of variables involved in SCP production in bubble column reactor: In this study, as done by other investigators (House and Place, 1972 and 1973; Harrison and Doddema, 1977) the type of media, nitrogen source, inoculum volume, and ratio of natural gas to air were selected as effective factors in the process of biomass production in the bioreactor. In order to determine the minimum number of experiments, *Automatic Design and Analysis of Taguchi Experiments* (Roy, 1990), was applied through Qualitek software (version IV). Table 1 inputs these variables to the software in three levels.

After 120h reaction for each of 9 experiments suggested by Taguchi Analysis Method, the biomass was separated immediately by centrifugation at 1410 $\times$g for 15 min. The supernatant was decanted and the sludge washed with distilled water and dried at 70°C and biomass weight in dry basis was determined after all.

Protein measurement and amino acids analysis: Protein quantity was measured using Micro Kjeldahl method (APHA *et al.*, 1992). The amount of total protein was determined by Lang method (Lang, 1958). An HPLC (Waters; 1525 binary HPLC pump; 2487 dual γ absorbance detector; 3.9*150 mm PICO.TAG Amino Acid Analysis Column; USA) equipped with a μ-BONDAPAK^{TM}/$_{C_{18}}$ column was used for qualitative and quantitative analysis of amino acids. The amino acid analyzing column functioned in three different temperature zones including 110, 38.5, and 25°C for executing different analyzing steps i.e. hydrolysis, separation, and derivation respectively. Using a standard chart graphed for known amino acids concentrations, quantity of each amino acid was calculated.

RNA measurements: For the optimum test (at the

Table 1. Variables and levels in the process of SCP production from natural gas.

Variables	Levels		
	1	2	3
Medium	MSB*	NMS**	MWC***
Nitrogen Source	$(NH_4)_2SO_4$	$NaNO_3$	Urea
Inoculum Volume	7.00	5.00	10.00
CH_4/Air	2.66	1.33	0.53

*　　　Methane Salt Broth
**　　Nutrient Mineral Salts
***　Medium Without Carbon

beginning of its log-phase) 24 samples (each 10 ml) were taken and exposed to different heat shocks for 10, 15, 20, 30 and 60 min at 55, 60, 65 and 70°C. Samples were shocked by a water bath and cooled down immediately to 4°C and kept at this temperature for one hour. After cooling process, they were centrifuged for 15 min at 1410 ×g. The supernatants were decanted and biomass was taken for RNA extraction. An RNX^{TM} (-Plus) solution (*CinnaGen Co. Iran*) with its extraction protocol was used for measuring the biomass RNA (Cox, 1968; Chomczynski and Sacchi, 1987; Chomczynski, 1993). 5 μl of the RNA solution diluted with 495 μl sterilized distilled water (dilution process for 100 folds). The optical density of the RNA extracted from biomass was then measured at 260 and 280 nm.

RESULTS

Results of Taguchi design method: The results of the experiments based on variables and levels are shown in Table 1 are illustrated in Figures 2, 3, and 4. These figures demonstrate the biomass growth based on the results of optical density for different cultures (MSB, NMS, and MWC) with different nitrogen sources at 600 nm. For each experiment, biomass specific growth rate and raw protein values were determined; results are shown in Table 2. Also the logarithmic phase time, as well as biomass dry weight for each of these 9 experiments are available in the same table.

The optimum condition (optimized parameter setting for highest biomass production): For getting the optimum condition, amounts of raw protein values shown in Table 2, along with each corresponding experiment specifications (medium, nitrogen sources, initial inoculum volume, and inlet gas mixture), were fed into the Taguchi software again. MSB as the best medium and sodium nitrate as the most appropriate nitrogen source were recognized by the software. Furthermore, 7% percent for each initial inoculation and a natural gas/air ratio equal to 1.50 as the best volume configuration of inlet gas mixture were chosen as the optimum conditions.
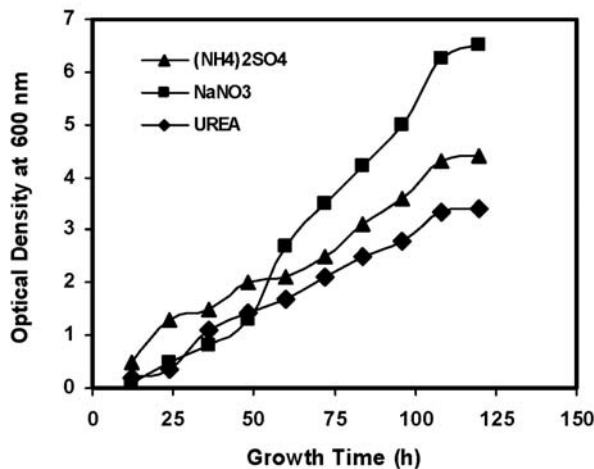

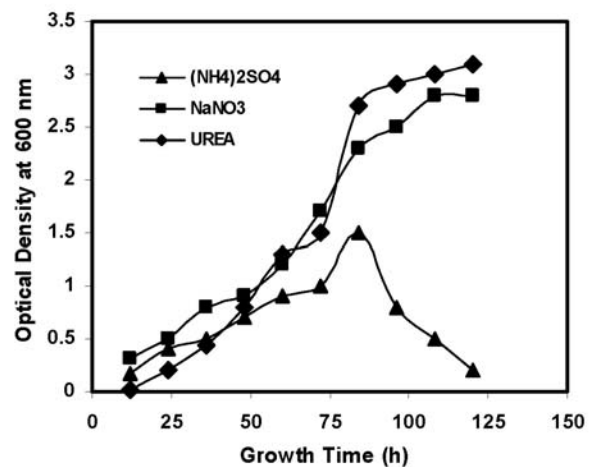**Figure 2.** Optical density of MSB culture with three different nitrogen sources at 600 nm.

Figure 3. Optical density of NMS culture with three different nitrogen sources at 600 nm.
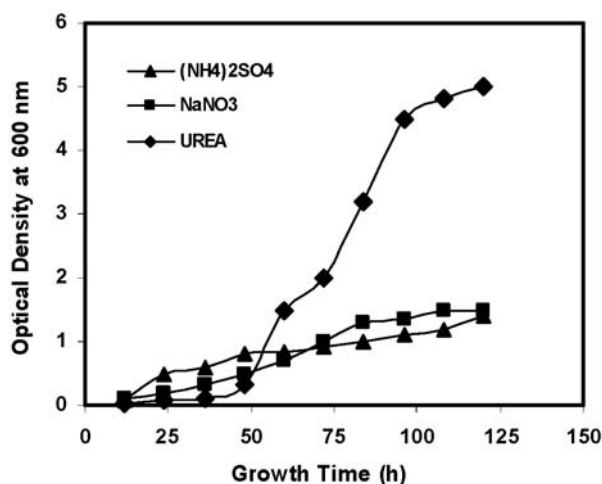
Figure 4. Optical density of MWC culture with three different nitrogen sources at 600 nm.

Figure 5. Optical density of microbial growth at 600 nm, when medium, nitrogen source, inoculum volume (size), and ratio of natural gas to air were selected as equal as results given by Taguchi optimization (MSB for medium, $NaNO_3$ for nitrogen source, 7 (v/v)% for inoculation size, and a flow rate ratio of 1.5 for natural gas to air).

DISCUSSION

From the results of Taguchi test, sodium nitrate was selected as the most appropriate nitrogen source. This fact, however, will be significant when MSB is used as the culture media (Fig. 2). Also as shown in Figure 3, when NMS used, sodium nitrate could be an effective nutrient for bacterial growth. Although urea demonstrated a higher ability in biomass growth in MWC and NMS media (Figs. 3, 4), it resulted in some low amounts of raw protein compared to sodium nitrate (Table 2). Ammonium sulfate gave undesired growth for cells (Figs. 3,4) and relatively low amounts of raw protein in most cases. Meanwhile in one case, even its presence, a phenomena like endogenous respiration was observed in a duration time about 75h (repeated 2 Times) (Fig. 3). However more research needs to explore this behaviour.
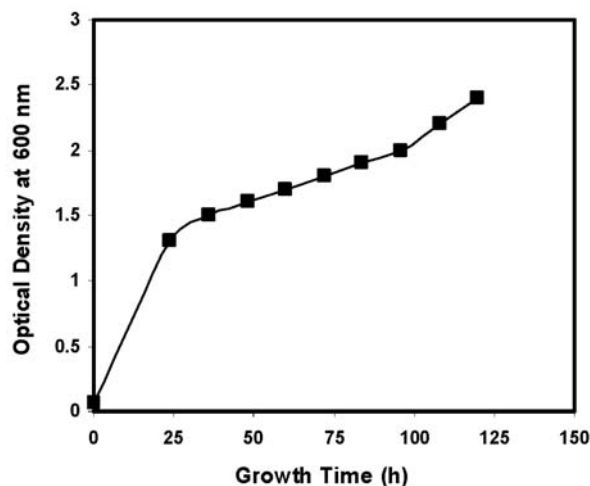
Figure 5 shows the optical density of the Taguchi optimum experiment at 600 nm. A growth phase about 110h is seen for this experiment which indicates the most optimum condition for cell growth. There was also a maximum raw protein quantity in this experiment equal to 69.3 % of the biomass dry weight.

For highlighting the nutrition value of the optimum produced biomass, quantitative and qualitative HPLC amino acid analysis was performed (Fig. 6). Based on the values extracted from HPLC, the concentration of amino acids in the biomass could be estimated by the standard chart (Fig. 6B). The quantities of amino acids obtained from biomass in other studies worked on natural gas, methanol, and other sources were compared in Table 3 (Gas as substrate: House and Place, 1972;

Table 2. Biomass specific growth rate and raw protein values based on the Taguchi method.

	Elements of Taguchi test used in this work				Logarithm phase time (h)	Biomass dry weight (g)	Raw protein (wt %)	μ^{**} (h⁻¹)
No.	Media	N. Source	Ino.* (v %)	CH_4/Air				
1	MSB	$(NH_4)_2SO_4$	7	2.66	84	1.80	58.5	0.014
2	MSB	$NaNO_3$	5	1.33	84	1.95	60.0	0.030
3	MSB	Urea	10	0.53	84	1.73	50.0	0.027
4	NMS	$(NH_4)_2SO_4$	5	0.53	48	1.65	33.3	0.028
5	NMS	$NaNO_3$	10	2.66	84	1.74	50.3	0.021
6	NMS	Urea	7	1.33	96	1.72	51.2	0.029
7	MWC	$(NH_4)_2SO_4$	10	1.33	96	1.55	45.0	0.012
8	MWC	$NaNO_3$	7	0.53	84	1.66	48.0	0.024
9	MWC	Urea	5	2.66	96	1.63	40.0	0.043

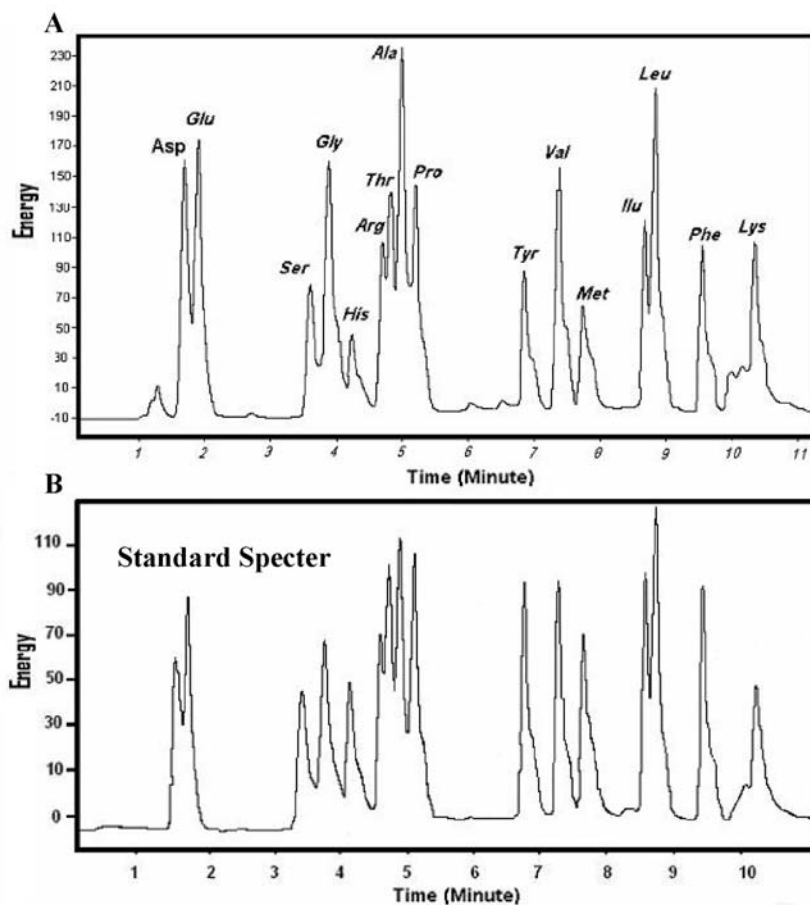*   Inoculum (v/v %)
**   Specific growth rate

Figure 6. The HPLC chromatograms of digested biomass optimized sample (**A**) and the standard chart (**B**).

Harrison and Doddema, 1977. Methanol as substrate: Peppler and Perlman, 1979. Other available protein sources such as fish meal and soybean: House and Place, 1972).

A general domination in the concentrations of microbial-source amino acids compared to other sources is seen by the Table 3. This ascendancy is appeared more amenable when it focused on three essential acids i.e. methionine, aspartic acid, and argenine. Although, lysine, histidine, and threonine are the only amino acid weakly displayed in this category, and their concentration seems to be suitable for feeding.

Table 4 shows the RNA concentration and raw protein before and after heat shock. The best RNA reduction result should be selected due to three main factors: a) RNA decom-position level, b) minimum protein lost, and c) low energy consumption. At 60 and 65°C, highest RNA reduction were occurred (results are bolded and underlined in Table 4). However, at the first glance, the best result for RNA level (0.70 μg/μl) could not be chosen for the final heat shock treatment due to its high energy consumption and more protein

loss (20%). Therefore, optimum heat shocks, well-matched to above mentioned factors, might be applied in the range of 60 to 65°C for a retention time between 10 to 20 min. The absorption ratio reads in 260 and 280 nm (A_{260}/A_{280}) gives the level of RNA extraction (Fleck and Munro, 1962); showing a degree of RNA purification from phases rich in DNA and proteins (this ratio is shown by "**D**" in Table 4). Ratios, guaranties acceptable selective RNA extraction, normally lie in the range of 1.85 to 2.15. Amounts extremely lower than this range, imply a contamination of the aqueous layer with bio-molecules in interphase (DNA-rich) or in the organic phase (proteins- rich). In our results, as Table 4 indicates, an average equal to 1.93 can be allocated for **D** value, which represents satisfactory results for RNA degree of purification.

Numbers were bolded and displayed by italic digits (Table 4) are the initial amounts of RNA existed in biomass bulk. Since these amounts were experimented before heat shock treatment, they could be addressed to the initial RNA potential of our isolated organisms. An average around 10 g of RNA out of any 100 g of

Table 3. Comparison of amino acid distributions of methane derived–protein with other protein sources (grams of amino acids in 100 grams of dried biomass).

Amino Acids	Produced Biomass (this work)	T_4*	999*	ATCC ** 21,310	Soya** Meal	Fish** Meal	Proteen*** ICI
Leucine	4.10	7.28	7.30	8.19	7.60	6.40	7.60
Valine	1.50	6.31	5.99	6.64	5.20	4.50	5.60
Lysine	5.90	5.49	5.24	5.87	6.60	6.70	6.80
Phenylalanine	2.50	4.59	4.65	4.81	4.80	3.40	3.60
Threonine	3.10	4.42	4.34	4.64	3.90	3.80	4.60
Isoleusine	—	3.83	4.21	4.57	5.80	3.80	4.80
Methionine	3.60	2.23	2.59	1.48	1.10	2.40	2.50
Cysteine	0.50	N/D	0.57	0.26	1.20	0.90	0.70
Tryptophane	0.50	—	—	3.81	1.20	0.90	1.00
Glutamic acid	10.50	9.39	9.39	11.15	18.50	13.40	10.50
Aspartic acid	11.20	8.55	8.29	9.57	—	8.60	9.00
Alanine	3.10	7.66	6.32	9.18	3.80	6.30	7.00
Serine	3.30	3.49	3.40	7.61	5.60	4.10	3.40
Glysine	3.20	5.41	4.68	6.05	8.30	9.30	5.30
Arginine	8.20	5.63	5.04	5.79	7.00	6.10	5.00
Proline	—	7.39	6.53	4.14	5.40	5.50	3.40
Histidine	2.30	2.07	2.06	2.38	2.50	2.00	2.00
Tyrosine	1.70	2.17	3.97	3.86	3.20	2.80	3.30

* Harrison and Doddema, 1977
** House and Place, 1972
*** Peppler H.J. and Perlman D,1979

cellular protein were extracted from our bio-sample. Bacterial-source proteins usually contains 8 to 25 g RNA in their 100 g weight (Abu-Ruwaida *et al.*, 1988). RNA content for animal feed does not cause to any crucial problem (Peppler and Perlman, 1979); however, when proteins are to be consumed by human, its RNA dosage should be considered, scaled, and measured severely. In a human-diet, consumption of RNA more than 2 g per day results in harsh deceases like kidney-stone and gout (Anupama and Ravindra, 2000). RNA content in the bacterial-source protein gained from this experimental work is extremely near to the possible minimum, which in its own position decreases process equipments and energy consumptions at down-stream processing.

Toxicity is the most important factor causes SCP could be used by animal or human. This is a vast area that needs vast researches and experimental works which must be performed prior to RNA evaluation. However, we did not focus on this point and since no firm result has been reported on toxicity of *Methylomonas*, we assumed on its safety and considered on the RNA content and the effect of heat shock treatment on RNA lowering.

CONCLUSION

For production of single cell protein from natural gas, the methane utilizing microorganisms (mostly

Table 4. The results of RNA reduction by heat shock treatment.

	RNA Concentration (g RNA/100 g protein) and Raw Protein (wt %) During Heat Shock Treatment											
	55(°C)			60(°C)			65(°C)			70(°C)		
Treatment Time (min.)	RNA	P**	D***	RNA	P	D	RNA	P	D	RNA	P	D
0*	*10.7*	69.1	2.00	*11.0*	69.2	1.90	*10.2*	69.2	1.90	*9.5*	69.0	1.86
10	5.5	68.5	2.10	2.5	67.9	2.00	<u>1.2</u>	67.4	2.05	3.7	67.7	1.80
15	2.0	64.3	1.85	1.7	67.1	2.15	2.5	66.9	2.00	5.3	65.8	1.95
20	4.2	62.7	1.70	<u>**0.80**</u>	65.2	2.00	<u>1.1</u>	65.0	1.92	2.8	62.1	1.90
30	2.0	60.0	1.90	1.6	59.8	2.01	1.6	59.1	1.80	1.8	58.5	1.95
60	1.8	57.1	1.97	1.9	58.0	1.90	<u>0.7</u>	56.2	1.85	1.6	53.6	1.90

* At zero time (without heat shock)
** Protein wt% in biomass
*** Degree of RNA purification in phase rich in DNA and protein

Methylomonas sp.) were chosen due to their higher growth rate characteristics. According to the Taguchi method applied on a bench-scale bubble column reactor used for gas-liquid interaction, the optimum experimental design was selected as: MSB as the medium, sodium nitrate as the nitrogen source, inoculation volume for 7 percent, and 60/40 flow rate ratio for natural gas/air streams. The quantity of protein in the optimum test was determined as 69.3% on the basis of bacterial dry weight. This amount of protein and its structural amino acids can be in challenge with other nutrient sources like fish meal and soy meal (Table 3). RNA content of the isolated bacteria was extremely near to the possible minimum which can be reduced effectively by heat shock treatment at 60 to 65°C for 10 to 20 min.

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