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Original Article

Cellular lipid production of a heterotrophic bacterium isolated from poultry processing wastewater

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Abstract

Cell growth and lipid production of a heterotrophic bacterium, R4.4 which accumulated the highest lipid content among 12 bacterial colonies being isolated from wastewater of a poultry processing plant in Thailand, were evaluated in this study. The culture was identified as *Aeromonas* sp. by 16S ribosomal DNA sequencing analysis.The highest lipid content was obtained when the cells were in the early stationary growth phase compared to the cells in the exponential and the late stationary phase. Over 50% of the fatty acid production by *Aeromonas* sp. KMITL-R4.4 were unsaturated fatty acids, including linoleic acid (C18:2, 43.2%), oleic acid (C18:1, 19.0%), and palmitoleic acid (C16:1, 8.2%). The culture had a preference for glucose and fructose as seen from the maximum biomass and lipid contents that were obtained. Among volatile fatty acid (VFA) species tested, acetic acid was the preferred substrate for lipid production but not favorable for cell growth. In addition, ammonium sulfate was found to be the best among nitrogen sources tested. The C/N ratio exerts significant impact on lipid production as seen from an increase of the lipid content from 10.8% to 18.2% by exposing the bacterial cells to a medium with lower nitrogen concentration (0.1g/l) and higher level of glucose (28 g/l).

Keywords: heterotrophic bacterium, lipid accumulation, medium components, microbial lipid

1. Introduction

The concept of using microbial lipid as the supplementary sources of conventional oils and fats has often been studied since the 1980s (Patnayak and Sree, 2005). Microorganisms are considered as abundant sources of oils and fats because their membranes and membranous structures always contain lipids (Zhang *et al.*, 2011). In addition, eukaryotic microorganisms including fungi and yeast as well as certain groups of prokaryotes such as *Arthobacter* sp., *Rhodococcus* sp. *Gordonia* sp. and *Streptomyces* sp. frequently accumulate large quantities of triacylglycerols (TAGs), which are non-polar and water-insoluble triesters of glycerol with fatty acids, during period of metabolic stress (Alvarez *et al.*, 2002; Gouda *et al.*, 2008; Kosa and Ragauskas, 2011). Furthermore, most bacteria, which are able to accumulate storage lipid, also produce specialized lipids, such as poly(3-hydroxybutyrate) (PHB) and other polyhydroxyalkanoates (PHA) as intracellular inclusions under some growth-restricted conditions (Packter and Olukoshi, 1995; Alvarez *et al.*, 1996). Investigation of the isolated lipids showed similarities in type and composition of microbial oils to vegetable oils and animal fats which proves them to be a convenient substitute for conventional oils in many applications including the production of pharmaceutically and neutraceutically important polyunsaturated fatty acids (PUFAs) (Patnayak and Sree, 2005) as well as a vehicle aids for medicament in therapeutic or pharmaceutical industry

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(Alvarez *et al.*, 2002).

Recently, utilization of microbial lipid as an alternative feedstock for the production of oleochemicals especially fatty acid methyl esters (FAMEs), which are also known as biodiesel, has drawn interest of scientists to heterotrophic oleaginous microorganisms (Li *et al.*, 2008; Meng *et al.*, 2009). This prompts scientists to devote their efforts not only to screen microorganisms which produce high lipid yields by utilization of inexpensive bio-based feedstocks (Voss and Steinbüchel, 2001; Gouda *et al.*, 2008; Li *et al.*, 2010; Queiroz *et al.*, 2011), but also to produce lipid in a reproducible, high quality and sustainable way (Kosa and Ragauskas, 2011).

To fulfill the latter task, several efforts have been conducted by determining the fundamental factors that control the lipid production by oleaginous microorganisms (Kosa and Ragauskas, 2011) as well as trying to modify and optimize cultivation parameters (Hassan *et al.*, 1996; Li *et al.*, 2007; Beopoulos *et al.*, 2009; Kurosawa *et al.*, 2010; Subramaniam *et al.*, 2010; Wu *et al.*, 2010; Zhang *et al.*, 2011). While heterotrophic bacteria have not been as extensively characterized with respect to their lipid and fatty acid content as other microbes, the available information nonetheless suggests that they can provide an abundant source of neutral lipids as well as specialized lipids (Alvarez *et al.*, 2000; Alvarez *et al.*, 2002; Patnayak and Sree, 2005). This paper presents the results of lipid production and fatty acid composition of *Aeromonas* sp. KMITL-R4.4, a heterotrophic bacterium which was isolated from poultry processing wastewater. In addition, influences of medium component on lipid production such as carbon and nitrogen sources and C/N ratio on growth and lipid production are also evaluated.

2. Materials and Methods

2.1 Source of bacterial isolation and culture condition

The bacterial isolate R4.4 was originally isolated from a polishing pond which is a part of a poultry processing wastewater treatment system as previously described by Ongmali *et al.* (2010). Stock cultures were maintained in LB agar at 4°C with a subsequent transfer to a fresh medium every month. Before use, the isolated bacterium was subcultured twice on LB agar, then cultivated in LB broth and incubated at 30°C for 48 hrs.

2.2 Bacterial identification by 16S ribosomal DNA sequencing analysis

16S ribosomal DNA nucleotide sequencing method was used for the identification of the bacterial isolate R4.4. Genomic DNA of the isolated bacterium was extracted using DNA Wizard® SV Genomic DNA Purification System (Promega, USA). About 1,500 bp of 16S ribosomal DNA was amplified by two universal primers; 27F (5'-AGAGTTTGAT CCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGA CTT-3') using genomic DNA as a template. The PCR product of 16S rDNA was sequenced by First BASE Laboratories, Malaysia. Nucleotide sequence was analyzed and compared with GenBank nucleotide sequence database using the Basic Local Alignment Tool (BLASTn).

2.3 Determination of growth characteristics and lipid content

Seed culture of bacterial isolate R4.4 was acclimated and grown in the synthetic wastewater, according to Lee *et al.* (2003), prior to conducting an experiment. Unless otherwise stated, the individual synthetic wastewater containing glucose and $(NH_4)_2SO_4$ as carbon and nitrogen sources, respectively, was inoculated with the acclimated seed cultures to reach an initial optical density at 600 nm (OD_{600}) of about 0.1 and then incubated at 30°C for 72 hrs in an incubator shaker at a shaking speed of 200 rpm. Time course of cell growth was measured in terms of OD_{600}. The supernatants of the culture broth were used for analysis of reducing sugar. Cells in three different growth phases were harvested by centrifugation and their dry weight was determined in lyophilized form (Ongmali *et al.*, 2010). The cells were lyophilized and stored in a desiccator until lipid content was determined. At the end of experiment, the fatty acid profile of the lipid was also determined.

2.4 Effect of medium components

Three medium components, carbon and nitrogen source and C/N molar ratio, were tested for their effect on cell growth and lipid production by *Aeromonas* sp. KMITL-R4.4. The seed cultures were first acclimated to synthetic wastewater with the wastewater compositions according to Lee et al. (2003). Before any experiment, the seed culture was grown in synthetic wastewater until an initial OD_{600} of about 0.100 was obtained. In order to test the effect of carbon source, glucose in the original synthetic wastewater was substituted by (i) fructose, (ii) lactose, (iii) sucrose, (iv) sodium acetate, (v) propionic acid, and (vi) butyric acid. The concentration of $(NH_4)_2SO_4$ and C/N molar ratio were fixed at 1 g/l and 20, respectively. In order to test the effect of nitrogen source, $(NH_4)_2SO_4$ in the original synthetic wastewater was substituted by (i) urea and (ii) $NaNO_3$ with glucose as carbon source and the C/N molar ratio was equal to 20. To test the effect of the C/N ratio, experiments were performed by (i) varying the concentration of $(NH_4)_2SO_4$ from 0.1 to 10 g/l with fixed concentration of glucose resulting in a decreasing C/N ratio from 200 to 2, and (ii) varying the glucose concentration from 4.5 to 35 g/l with a concentration of $(NH_4)_2SO_4$ fixed at 0.1 g/l to yield a C/N molar ratio varying from 100 to 800. The flasks cultures were then incubated in an incubator shaker at 200 rpm and 30°C.

2.5 Analytical methods

Biomass as gram dry cell weight per liter of synthetic

wastewater was determined using freeze-drying and gravimetric technique. Analysis of reducing sugar was conducted by the Somogyi-Nelson Method. Lipids were extracted from lyophilized cells using chloroform and methanol according to Bligh and Dyer (1959). The mixture was centrifuged to obtain a clear supernatant and the solvent was removed by a rotary evaporator. Lipid content was expressed as gram lipid content per gram dry cell weight. The fatty acid profile of the lipid was determined as fatty acid methyl esters (FAMEs) and was analyzed by an Agilent 6850 gas chromatograph equipped with a flame ionization detector (FID).

3. Results and Discussion

3.1 Bacterial Identification

In our previous screening work, twelve bacterial colonies were isolated from water samples obtained from a poultry processing wastewater and the isolate R4.4 was found to accumulate the highest cellular lipid content (Ongmali *et al.*, 2010). Therefore, the bacterial isolate R4.4 was selected as a model microorganism in this study. For strain identification, the PCR product of 16S rDNA was amplified by using genomic DNA of the bacterial isolate R4.4 as a template and was subsequently sequenced. Nucleotide sequence of partial 16S rDNA was deposited in the Genbank under accession number JX546610. Nucleotide sequence analysis of 16S rDNA using BLASTn program revealed that the isolated bacterium R4.4 showed the highest homology to *Aeromonas sobria*, *A. hydrophila*, *A. veronii,* and *A. calicicola* (Table 1). Therefore, the bacterial isolate R4.4 was called *Aeromonas* sp. KMITL-R4.4, as the available information was insufficient for an assignment of the species.

3.2 Growth and lipid content of *Aeromonas* sp. KMITL-R4.4

As seen from Figure 1, an optical density at 600 nm (OD_{600}) increased from an initial value of 0.100 to approximately 4.0 with a decrease of glucose from 7.7 g/l to 2.9 g/l after 36 hrs of cultivation. These observations indicated the ability of *Aeromonas* sp. KMITL-R4.4 to grow in the synthetic wastewater containing glucose and $(NH_4)_2SO_4$ (initial C/N ratio = 20). During the first 36 hrs of cultivation, cells utilized carbon sources not only for cell proliferation, but also for the biosynthesis and accumulation of the important biomolecule, such as lipids, which can be seen from an increase of the biomass and lipid content from 0.31 g/l and 8.8% after 18 hrs of cultivation to 1.1 g/l and 11.6% after 36 hrs of cultivation (Figure 1). After 36 hrs of cultivation, the cells entered the stationary phase with a maximum OD_{600} of approximately 4.0 and had a maximum lipid content of 11.6%.

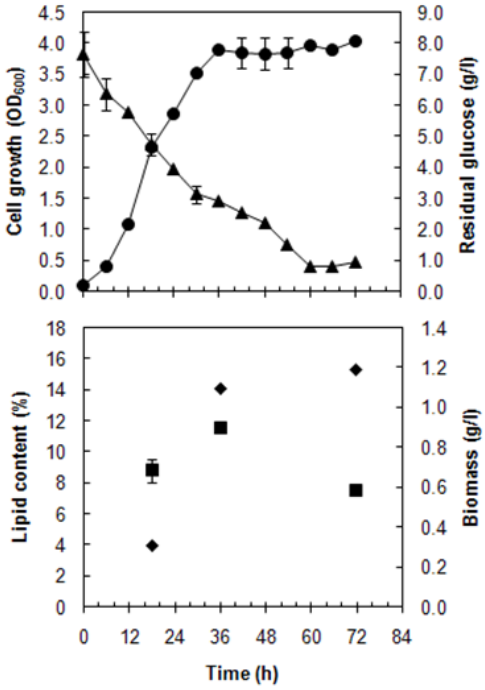

Figure 1. Time course of cell growth (●), glucose utilization (▲), lipid content (■) and biomass (◆) by cells of *Aeromonas* sp. KMITL-R4.4 cultivated in synthetic wastewater with C/N molar ratio of 20.

Table 1. Nucleotide sequence comparison of the bacterial isolate R4.4 with other nucleotide sequence database of Genbank using BLASTn.

No.	Name	Accession no.	Homology (%)
1	*Aeromonas sobria* strain JY081016-1	GQ232759	99.90 (1030/1031)
2	*Aeromonas sobria* strain NQ090701	HM358836	99.71 (1029/1032)
3	*Aeromonas sobria* strain LD081008A-1	GQ205446	99.71(1030/1034)
4	*Aeromonas hydrophila* NBRC 12658	AB680307	99.71(1034/1037)
5	*Aeromonas veronii* strain G10-2A	JN644562	99.71(1034/1037)
6	*Aeromonas veronii* strain BMA12_2B	JN644542	99.71(1034/1037
7	*Aeromonas veronii* strain JF4612	JN120274	99.71(1043/1046)
8	*Aeromonas veronii* strain YA090911	GU735964	99.71(1034/1037)
9	*Aeromonas veronii* strain CYJ202	FJ940848	99.71(1034/1037)
10	*Aeromonas calicicola* strain 03037TRG	AY347679	99.61(1033/1037)

During the cultivation period between 36 hrs and 72 hrs, biomass remained nearly constant whereas the lipid content dropped to 7.5% suggesting the possible exhaustion of carbon source in the growth environment and metabolization of stored lipids. Similar changes were also observed in fatty acid content of *Rhodococcus opacus* PD630 grown under the optimized growth conditions in batch culture fermentation (Kurosawa *et al.*, 2010) as well as *R. opacus* PD630 cultivated in mineral salt medium containing 0.05 g/l ammonium chloride and 1%(w/v) sodium gluconate (Alvarez *et al.*, 2000). GC analysis of lipid profile from cells harvested at 72 hrs post-inoculation showed that the accumulated fatty acids consisted of primarily unsaturated fatty acids including linoleic acid (C18:2, 43.2%), oleic acid (C18:1, 19.0%), and palmitoleic acid (C16:1, 8.2%) (Figure 2).

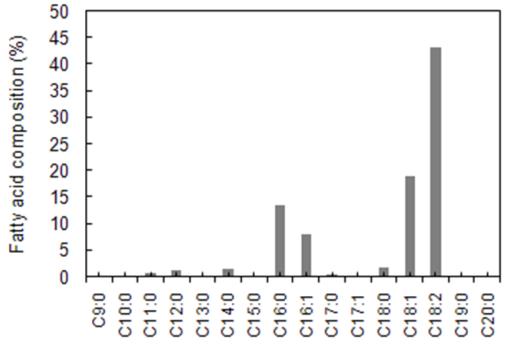

Figure 2. Fatty acid composition in the lipids extracted from *Aeromonas* sp. KMITL-R4.4 after cultivation in synthetic wastewater with C/N ratio of 20 for 72 hrs.

3.3 Effect of carbon sources

As depicted in Table 2, carbon sources exert significant influences on cell growth of *Aeromonas* sp. KMITL-R4.4. A high cell density as well as cell dry weight was obtained with glucose or fructose as the carbon source. In addition, cells cultivated in a medium containing glucose gave lipid contents (10.5±1.2%) that were not statistically significant different from the cells that were grown in a medium supplemented with fructose (12.0±0.9%). Quite poor biomass and lipid content were achieved from sucrose, while lactose was an unfavorable carbon source for cell growth and lipid production by *Aeromonas* sp. KMITL-R4.4. The differences between these sugars are thought to be attributed to the molecular size of the compounds corresponding to the ability of microbial cells to uptake and utilize carbon sources. While glucose and fructose is easily taken up by microbial cells, disaccharides like sucrose or lactose must be first hydrolyzed to monosaccharides or must have specific transport systems necessary before entering microbial cells as advocated by Perez-Garcia *et al.* (2011). In addition, poor lactose utilization may be related to a low lactase activity of microorganisms as well as cellular inability to utilize galactose easily. Among the three volatile fatty acids (VFAs) being studied, the highest biomass (0.35 g/l) and lipid content (12.1±1.0%) were observed when acetic acid was used as a carbon source. This finding is in agreement with previous reports by du Preez *et al.* (1996) and by Fei *et al.* (2011) who observed that acetic acid is the preferred VFA species for lipid production. A possible explanation for the preferential use of acetic acid over other organic acids revolves around a simple metabolic pathway for degradation of acetic acid which is degraded directly by the β-oxidation process forming acetyl–CoA (Elefsiniotis *et al.*, 2004). Comparing to other carbon sources like sugars, sodium acetate provided the cells with high lipid content that was not statistically significant different from the values obtained from fructose and

Table 2. Effect of carbon and nitrogen sources on cell growth and lipid production by *Aeromonas* sp. KMITL-R4.4 after cultivation in synthetic wastewater for 36 hrs.

Parameter	OD_{600}	Biomass (g/l)	Lipid content (%)
Carbon source			
Glucose	4.254±0.023	0.73	10.5±1.2
Fructose	4.668±0.042	0.72	12.0±0.9
Sucrose	3.338±0.176	0.612	8.2±0.7
Lactose	0.182±0.025	ND†	ND†
Sodium acetate	1.212±0.056	0.35	12.1±1.0
Propionic acid	0.120±0.000	ND†	ND†
Butyric acid	0.150±0.000	ND†	ND†
Nitrogen source			
$(NH_4)_2SO_4$	4.199±0.080	0.73	15.0±0.8
Urea	3.124±0.061	0.43	11.5±1.1
$NaNO_3$	0.307±0.036	ND†	ND†

† ND = Not determined due to low cell concentration.

glucose. However it was not a good carbon source in terms of cell growth as indicated by the low optical density (OD_{600} =1.212) and low cell dry weight (0.35 g/l).

3.4 Effect of nitrogen sources

Results presented in Table 2 show that $(NH_4)_2SO_4$ is the best nitrogen source for cultivation of *Aeromonas* sp. KMITL-R4.4 as indicated by the high optical density (OD_{600} = 4.199), high cell dry weight (0.73 g/l), and high lipid content (15.0±0.8%). Smaller amounts of biomass and lipid concentrations were achieved when cells were grown using urea as nitrogen source. Furthermore, $NaNO_3$ was not a suitable nitrogen source for cell growth and lipid production for *Aeromonas* sp. KMITL-R4.4. This finding suggests that *Aeromonas* sp. KMITL-R4.4 had the ability to utilize inorganic nitrogen sources, particularly in the ammonium forms, for cell growth and lipid production. Preferential utilization of ammonium over nitrate as a source of nitrogen by many organisms in the cell growth is due to the energy cost required for ammonium assimilation, which is lower than that of nitrate (Bloom *et al.*, 1999). In addition, the utilization of urea as a nitrogen source requires urease activity in cells in order to hydrolyze urea to ammonium which subsequently incorporated into cellular components (Paustain, 2002).

3.5 Effect of C/N ratio

Previous reports have demonstrated that the lipid production by oleaginous microorganisms is heavily influenced by the carbon-to-nitrogen (C/N) molar ratio (Koike *et al.*, 2001; Kurosawa *et al.*, 2010; Wu *et al.*, 2011). As shown in Figure 3A, when the $(NH_4)_2SO_4$ was increased from 0.1 to 10 g/l corresponding to a decrease of the C/N ratio from 200 to 2, the optical density increased from 2.1 to approximately 4.0. After 36 hrs of cultivation, higher amounts of nitrogen

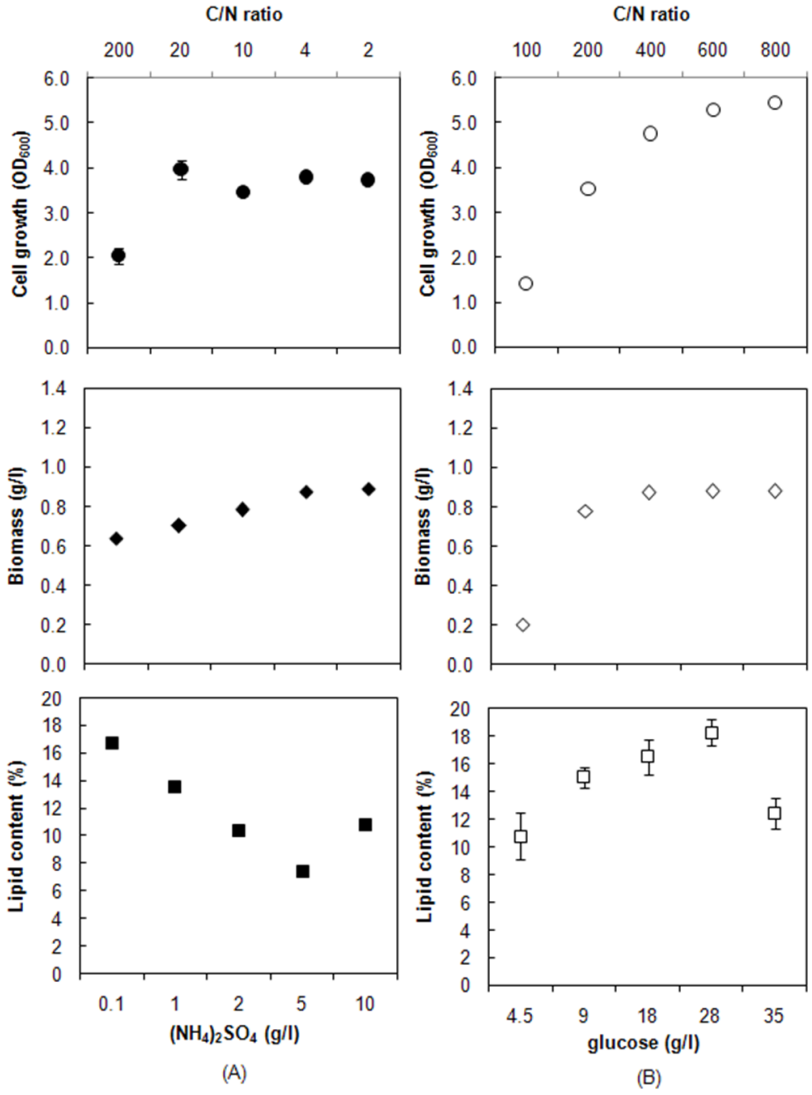

Figure 3. Effect of altering the C/N ratio on cell growth and lipid production by *Aeromonas* sp. KMITL-R4.4 during cultivation in synthetic wastewater at (A) different $(NH_4)_2SO_4$ concentrations and (B) different glucose concentrations for 36 hrs.

led to an increase in the biomass, with the highest cell dry weight of 0.89 g/l obtained when cells were cultivated in synthetic wastewater containing an initial $(NH_4)_2SO_4$ concentration of 10 g/l (Figure 3A). The results provide a strong indication of the importance of nitrogen level for bacterial cell growth. However, an increase in the concentration of $(NH_4)_2SO_4$ in the synthetic wastewater led to a decrease in the lipid contents of cells. *Aeromonas* sp. KMITL-R4.4 had the highest lipid content when cultivation with an initial $(NH_4)_2SO_4$ concentration of 0.1 g/l (Figure 3A). This observation supports the previous reports that an increase in $(NH_4)_2SO_4$ concentrations was favorable for cell growth but not suitable for lipid production (Alvarez *et al.*, 2000; Leesing and Nontaso, 2011).One possible explanation for a significant decrease in cellular lipid production is related to a pH drop in the culture supernatant as demonstrated by the work of Kurosawa *et al.* (2010).

Under limited quantity of nitrogen, an increase in glucose concentration relative to C/N molar ratio also affected both cell growth and lipid production by *Aeromonas* sp. KMITL-R4.4 as shown in Figure 3B. Concentrations of the cell dry weight increased from 0.20 to 0.88 g/l with an increase in the glucose concentration up to a limit of 18 g/l corresponding to a C/N molar ratio of 400. Once the glucose concentration exceeded this limit, a level off in cell dry weight was observed (Figure 3B). Similarly, the lipid content increased from 10.8% to 16.5% when the glucose concentration increased from 4.5 to 18 g/l, corresponding to an increase of the C/N ratio from 100 to 400. The lipid content reached the maximum value of 18.8% when the glucose concentration was at 28 g/l or C/N molar ratio was at 600. This result demonstrated that cultivation under excess of carbon and strict nitrogen-limiting conditions promotes lipid accumulation in the cytoplasm of bacterial cells as also reported in earlier investigations (Patnayak and Sree, 2005; Kurosawa *et al.*, 2010). A further increase in the C/N ratio beyond this value led to a clear drop in the lipid content. This observation is similar to what has been observed by other works wherein high concentration of glucose is speculated to be responsible for an inhibitory effect on microbial cell growth and subsequently microbial lipids (Riesenberg and Guthke, 1999; Li *et al.*, 2007; Leesing and Nontaso, 2011; Wu *et al.*, 2011).

As a summary, the above presented data and information provide an insight into the modification of culture conditions that result in high lipid contents of *Aeromonas* sp., a heterophic bacterium that was isolated from poultry processing wastewater.

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