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The effect of carbon and nitrogen sources on the fatty acids profile of *Mortierella vinacea*

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Abstract

Introduction: Microbial lipids attract attention of many researchers due to their therapeutic effects. The goal of this study is the production and optimization of lipids and fatty acids in *Mortierella vinacea* by applying different media to achieve invaluable fatty acids in pharmaceutical and food industry.

Materials and methods**:** *Mortierella vinacea* was cultured on potato dextrose agar. Then the spores were inoculated to the production medium. After 72 hours, the lipids were extracted and they were analyzed by gas chromatography. To optimize lipid and important fatty acids production in medium, various carbon and nitrogen sources were substituted with glucose and yeast extract respectively.

Results: The effect of some carbon and nitrogen sources on biomass, lipid and fatty acids production were assayed. The highest level of lipid production was in a medium which contains lactose and yeast extract (26.66%). Linoleic acid was only produced in presence of lactose and yeast extract (25.7%). While, *M. vinacea* yielded the highest level of linoleic acid (52.76%) in a medium containing peptone, linolenic acid was achieved only in presence of lactose and triptone.

Discussion and conclusion: In this study, lactose as a carbon source was the most effective one in the production of lipids. In addition, linoleic acid was produced in presence of lactose, so lactose was selected as the best carbon source. Peptone and triptone as a nitrogen source were chosen for the production of linoleic acid and linolenic acid in *M. vinacea* respectively. All of these findings reveal that *Mortierella* strain is a potential candidate for enhancement of linoleic acid and linolenic acid production. Furthermore, this simple media can be used in production of linoleic acid and linolenic acid for industrial goals in large scales.

Key words: Fatty acid, Lipid extraction, Gas chromatography, *Mortierella vinacea*

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Introduction

Lipid biotechnology is currently emerging as a significant field due to numerous applications of fatty acid and lipid derivatives in various applications such as food and pharmaceutical industries. The n-3 and n-6 (for example linolenic acid and linoleic acid, respectively) chains of polyunsaturated fatty acids (PUFAs) are introduced as potential food additives or pharmaceuticals for their biological activities. The PUFAs can be applied for the treatment of cardiovascular illnesses, cancers and as a participant in the inflammatory reactions (1-9).

Oleaginous microorganisms, such as microalgae, yeasts, fungi and bacteria can produce high levels of lipids and do not need arable lands. The fatty acid profiles are dependent on oleaginous microorganism's types and the growth conditions. Environmental conditions such as temperature, pH, substrate, C/N ratio and oxygen pressure have an effect on the productivity of accumulating lipids. Accumulation of lipids in yeasts and fungi chiefly is deliberate because these microorganisms often produce polyunsaturated fatty acids (10-15).

There are various reports in the literature for lipid production in *Mortierella*. For example, it has been reported that total lipid in *Mortierella alpina* was 36%. In another research by Kimura et al, the total lipid level in *Mortierella isabellina* was 33%. In both of the trials, glucose was selected as a carbon source. In another study, the concentration of linoleic acid was reported 9.8% in media by *Mortierella alliacea* and percentage of linolenic acid was 3.4% (14-16). However, the outstanding emphasis of this study is improvement and production of lipids and fatty acids in *Mortierella vinacea* PTCC 5262, using suitable substrates at the optimum conditions.

Materials and methods

Chemicals: All solvents, chemicals and carbohydrates were purchased from Merck Company. Mineral salts were prepared from Sigma Company.

Culture conditions: *Mortierella vinacea* PTCC 5262 was purchased from Iranian Research Organization for Science and Technology (IROST) and it was applied for fatty acid production. *Mortierella vinacea* was maintained on potato dextrose agar (PDA) slants with repeated sub-culturing for its viability. The culture was incubated in 250 ml Erlenmeyer flask for 72 hours at 28°C on a controlled rotary shaker at 180 rpm. The production medium (basal medium, BM) contained (g/l): glucose 40, yeast extract 1.5, NH_4NO_3 0.286, KH_2PO_4 0.75, $MgSO_4 \cdot 7H_2O$ 0.4 and $CaCl_2$ 0.4 (pH = 5.6). The flask containing 50 ml culture medium was inoculated with suspension containing 1×10^8 spores/ml. The total number of spores was determined by counting under microscope using haemocytometer (Neubauer counting chamber). The fungal spore count was adjusted to 1×10^8 spores/ml by making

appropriate dilutions (17).

Determination of biomass: Biomass was obtained from culture broth through Whatman No. 1 filter paper. Then it was washed twice with distilled water and it was dried at 60°C for sufficient time to reach to a fixed weight (usually after 24h). The dry cell weight was determined gravimetrically (18, 19).

Lipid extraction: The produced lipids were extracted from mycelia according to a modified procedure by Bligh and Dyer. Briefly, cells were accumulated through Whatman No.1 filter paper, then the fungi was washed twice with distilled water, then 1g of cells were added to 10 ml of 4 M HCl, and they were incubated at 60°C for 2h. The acid hydrolyzed mass was shaken with 20 ml of chloroform/methanol mixture (1:1) at room temperature for 2h to 3h, followed by centrifugation at 2000 rpm for 5 min at room temperature using Sigma 3K30 centrifuge in order to separate the aqueous upper phase from organic lower phase. The upper phase was removed by a Pasteur pipette and the lower phase containing lipids was gathered by a sampler and then it was evaporated under a reduced pressure for 10 min (19).

Fatty acid analysis

Preparation of fatty acid methyl esters (FAMEs): Free fatty acids were esterified and then o-acyl lipids were transesterified by heating them with a large excess of anhydrous methanol in presence of an acidic catalyst. Fatty acids were methyl esterified according to the procedure by Christie. The sample of lipid (50 mg) was dissolved in a test tube fitted with 1 ml toluene by a condenser. Then 2ml 1% sulfuric acid in methanol was added and this solution was maintained at 50°C for 24h. The methylated fatty acids were separated from the aqueous layer by adding 5ml of 5% NaCl and then it was dissolved in 2×5ml n-hexane. The n-hexane layer was separated by Pasteur pipettes, which was washed with 4 ml of water containing 2% potassium bicarbonate. Then it was dried over anhydrous sodium sulfate. The solution is filtered and the solvent was removed under reduced pressure in a rotary film evaporator or in a stream of nitrogen (20).

Fatty acids content determination by gas chromatography (GC): GC was performed on Agilent 19091J-413 Series Gas Chromatograph which is equipped with a FID and the capillary column HP5 (30 m ´ 0.25 mm i. d., 0.25 µm film thickness; USA). Injector and detector temperatures were maintained at 260°C and 300°C, respectively. The oven was programmed at 100°C for 2 min, and then it was increased to 160°C at 3 min. Therefore, it was maintained for 2 min at 215°C, it was increased more to 217°C at 2 min, then it was maintained at 218°C for 2 min and finally it was increased to 260°C at 2 min. The carrier gas, nitrogen, was used at a flow rate of 1.5 ml/min. The injection volume was 1 µL, with a split ratio of 50:1.

Determination of glucose concentration: The glucose concentration was estimated with the dinitrosalicylic acid assay in the medium according to a method described by Xiong et al. (21).

Effect of different carbon sources on lipid and fatty acid content: Glucose in the basal medium was replaced by sucrose, fructose and lactose and the medium was incubated at 28°C for 72h. 40 g/l of the carbon source was added to the basal medium.

Effect of different nitrogen sources on lipid and fatty acid content: Nitrogen source in the basal medium was replaced by 1.5% of peptone and triptone at 28°C for 72h. Lactose (40 g/l) was consumed as a carbon source in both of them.

Results

Growth characteristics and lipid production of *M. vinacea*: Lipid production of the fungal cells was increased gradually and then it was declined at 96h. Maximum yield of lipid was observed in the beginning of the stationary phase (4.76% in 72h). The initial glucose concentration was 40 g/l which was consumed during the cell growth and lipid accumulation and it was depleted in 96h. Value of the initial pH in all tests was 5.5-5.8 (Fig. 1).

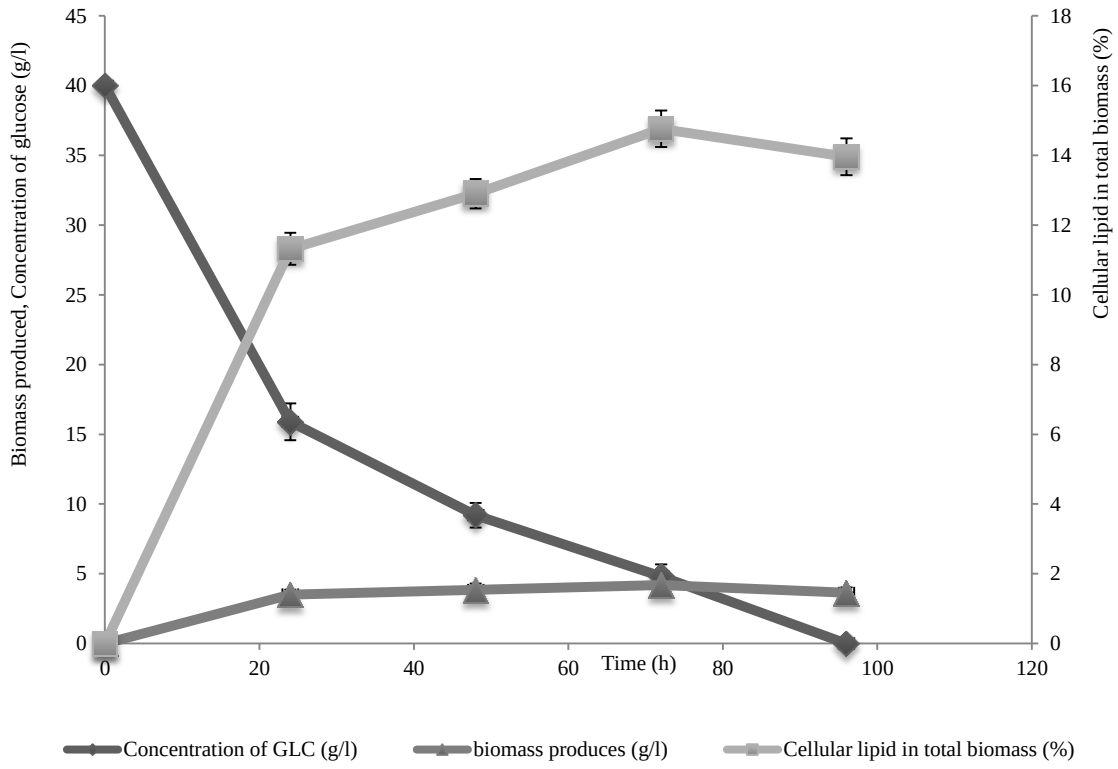

Fig. 1- Kinetics monitoring of biomass (g/l), cellular lipid in total biomass (%) and glucose concentration (g/l), in *Mortierella vinacea* PTCC 5262 on the medium. Medium conditions: initial glucose concentration 40 g/l, initial yeast extract concentration 1.5 g/l, initial pH = 5.6,T =28°C, Incubation Time 96 h. Data are presented as mean values from triplicate experiments.

Effect of different carbon sources on lipid and fatty acid content of *M. vinacea*: The effect of several carbon sources on biomass and lipid production was investigated (Table 1)**.** The best cell growth was obtained by fructose followed by sucrose, glucose and lactose. Although, higher quantities of lipid in absolute values (L_{max}, g/l), biomass (x, g/l) and total biomass yield on the consumed carbon source ($Y_{X/S}$, g/g) were produced on fructose more than on lactose (0.84, 6.4 and 0.16 against 0.32, 1.2 and 0.03g/l respectively) during growth. Cellular lipid in total biomass ($Y_{L/x}$, %) was 13.12 % in the fructose. But, the highest level of $Y_{L/X}$ was found in the media containing lactose and yeast extract (26.66 %).

The lipid yield on the consumed carbon source ($Y_{L/S}$, g/g) was equal in the presence of glucose, sucrose, fructose in the media (also the media contained lactose and peptone). In all of the tested carbon sources, palmitic acid (16:0) was produced, but stearic acid (18:0) was only found in the presence of glucose and fructose. When the media contained lactose, sucrose, fructose and glucose, oleic acid (18:1) contents of 9.18, 9.82, 18.41 and 44.11% were obtained respectively. However, the maximum oleic acid yield was detected by glucose. Linoleic acid was produced only in the presence of lactose and its level was 25.7%. Therefore, lactose was selected as a carbon source in the following experiments.

Table 1- Growth and fatty acid composition of *Mortierella vinacea* PTCC 5262 in nitrogen limited media with carbon based sources as substrates.

								Fatty acids (%)			
Carbon source	Nitrogen source	X (g/l)	$Y_{X/S}$ (g/g)	L_{max} (g/l)	$Y_{L/S}$ (g/g)	$Y_{L/X}$ (%)	C16:0	C18:0	C18:1 (n-9)	C18:2 (n-6)	C18:3 (n-3)
Glucose	Yeast extract	4.2	0.11	0.62	0.02	14.76	18.23	11.05	44.11	-	-
Sucrose	Yeast extract	4.4	0.11	0.64	0.02	14.54	5.35	-	9.82	-	-
Fructose	Yeast extract	6.4	0.16	0.84	0.02	13.12	9.12	5.53	18.41	-	-
Lactose	Yeast extract	1.2	0.03	0.32	0.01	26.66	14.70	-	9.18	25.70	-
Lactose	Peptone	4.6	0.12	0.62	0.02	18.70	26.47	-	13.64	52.76	-
Lactose	Triptone	2.6	0.07	0.46	0.01	17.69	10.23	-	-	-	20.00

Strain PTCC 5262 was cultivated in a medium containing 40 g/l carbon sources and 1.5 g/l nitrogen sources, pH 5.6, with shaking at 28°C for 72h. Representation of biomass (X, g/l), total biomass yield on the consumed carbon source ($Y_{X/S}$, g/g), cellular lipid in total biomass ($Y_{L/X}$, %) and lipid yield on the consumed carbon source ($Y_{L/S}$, g/g) when the maximum quantity of cellular lipids (L_{max}, g/l) was achieved. C16:0, palmitic acid; C18:0, stearic acid; C18:1(n-9), oleic acid; C18:2(n-6), linoleic acid; C18:3(n-6). Minor fatty acid components are not shown. Data are presented as mean values from triplicate experiments conducted by using different inocula.

Strain PTCC 5262 was cultivated in a medium containing 40 g/l carbon sources and 1.5 g/l nitrogen sources, pH 5.6, with shaking at 28°C for 72h. Representation of biomass (X, g/L), total biomass yield on the consumed carbon source ($Y_{X/S}$, g/g), cellular lipid in total biomass ($Y_{L/X}$, %) and lipid yield on the consumed carbon source ($Y_{L/S}$, g/g) when the maximum quantity of cellular lipids (L_{max}, g/L) was achieved. C16:0, palmitic acid; C18:0, stearic acid; C18:1(n-9), oleic acid; C18:2(n-6), linoleic acid; C18:3(n-6). Minor fatty acid components are not shown. Data are presented as mean values from triplicate experiments conducted by using different inocula.

Effect of different nitrogen sources on lipid and fatty acid content of *M. vinacea*: Utilization of some nitrogen sources by *M. vinacea* was investigated (Table 1). The best cell growth among other nitrogen sources (yeast extract, triptone and peptone) was peptone. In all of the tested nitrogen sources, palmitic acid was produced, but stearic acid was not detected in all of them. Linoleic acid and oleic acid were produced in the media containing yeast extract and peptone, whereas there were not any linoleic acid and oleic acid in the presence of triptone (Fig. 2). *M. vinacea* produced the highest level of linoleic acid (52.76%) in the medium that contained peptone. Linolenic acid was only obtained in the presence of triptone. Hence, peptone and triptone were chosen as nitrogen sources for *M. vinacea*.

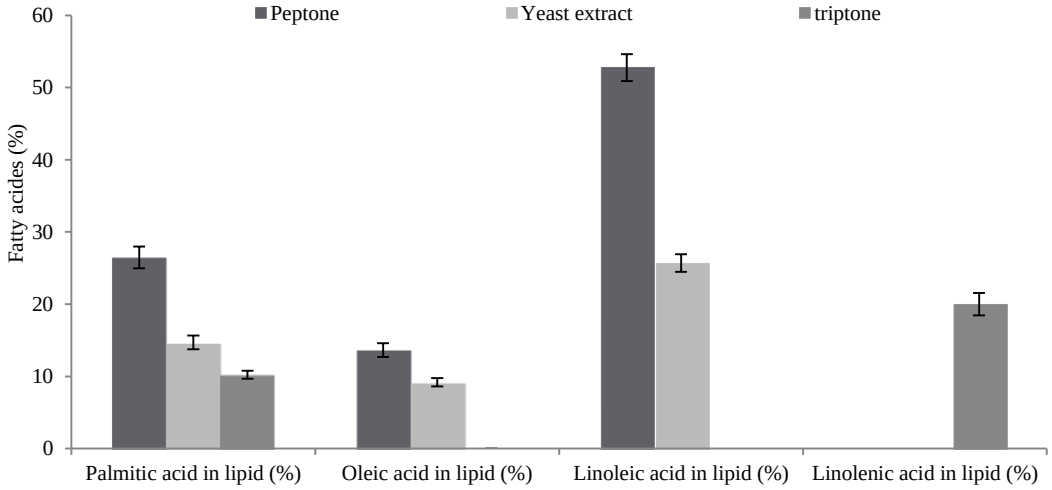

Fig.2- Fatty acid composition (%) of the *Mortierella vinacea* PTCC 5262 lipid produced in different nitrogen sources (1.5 g/l) in the medium. Lactose (40 g/l) used as a carbon source in all of them (Incubation Time, 72h). Data are presented as mean values from triplicate experiments conducted by using different inocula.

Discussion and conclusion

In oleaginous microorganisms, the lipid accumulation begins when carbon source exists in excess and an essential element in the growth medium becomes limited. Among known limitations for inducing lipid accumulation, usually nitrogen limitation is used for this purpose because it is the most efficient type of limitation. The assimilation of carbon source by organisms continues in the nitrogen limited conditions but proliferation stops because nitrogen is required for the protein and nucleic synthesis (13, 14, 22-27).

In the current study, the percentage of lipid production was 26.66% by *M. vinacea* in the presence of lactose and yeast extract (Table 1). Kimura et al. and Certik and Shimizu, also reported similar results for other strains of *Mortierella*. However, other sources of carbon and nitrogen (for example: glucose, peptone and malt extract) were applied for these tested strains (28, 16).

Jang et al. found that, soluble starch and glucose increased lipid production and arachidonic acid is the predominant cellular fatty acids in *Mortierella alpina* ATCC 32222. This result was similar to another study conducted by Chen and Chang on

Mortierella alpina CBS 210.32, and Shinmen et al. on *Mortierella alpina* IS-4. In addition, Rocky Salimi et al. selected glucose and yeast extract as the most suitable carbon and nitrogen sources for the optimization steps, respectively. This is in agreement with the findings of other researchers (18, 29-33). Chen and Liu reported that *C. echinulata* cultivated on various carbon sources produces high biomass quantities but the fungus did not accumulate large amounts of storage lipids (34).

Papanikolaou et al. reported glucose as the best carbon source in *Mortierella isabellina*. Aki et al. selected glucose and yeast extract as the best carbon and nitrogen sources, respectively in *Mortierella alliacea* YN-15 and arachidonic acid was the predominant fatty acids (35, 36). In this study, lactose as a carbon source had the maximum effect on lipid production. In addition, linoleic acid was produced in the presence of lactose, so it was chosen as the best source of carbon. Using lactose as a carbon source and peptone as a nitrogen source enhanced the linoleic acid production to 52.76%. While triptone was used as a nitrogen source, linolenic acid was produced, with no trace of linoleic acid (Table 1). In addition, linoleic and linolenic acids were predominant fatty acids which were produced by *M. vinacea*. By contrast, other studies reported arachidonic acid as predominant fatty acids in various strains of *Mortierella* (18, 29, 30, 35). All these findings suggest that the present *Mortierella* isolate is a potential candidate for further strain improvement to enhance the production of linolenic and linoleic acid content. This simple condition of culture media can be applied for large-scale production of linoleic and linolenic acids, which are suitable for pharmaceutical and nutritional industries.

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اثر منابع کربنی و نیتروژنی بر روی پروفایل اسیدهای چرب قارچ *مورتیرلا وینشیا*

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چکیده

مقدمه: لیپید میکروبی به علت خواص درمانی اسیدهای چرب، توجه پژوهشگران را به طور گسترده‌ای جلب کرده است. هدف این مطالعه تولید و بهینه‌سازی لیپیدها و اسیدهای چرب توسط قارچ *مورتیرلا وینشیا*، با استفاده از محیط‌های کشت مختلف بود تا اسیدهای چرب با ارزش برای استفاده در صنایع غذایی و دارویی به دست آید.

مواد و روش‌ها: قارچ *مورتیرلا وینشیا* روی محیط کشت پوتیتو دکستروز آگار کشت داده شد. سپس اسپورها به محیط تولید تلقیح داده شدند. بعد از ۷۲ ساعت، لیپیدها استخراج و توسط کروماتوگرافی گازی آنالیز شدند. برای بهینه‌سازی تولید لیپید و اسیدهای چرب مهم در محیط کشت، منابع کربنی و نیتروژنی مختلفی به ترتیب جایگزین گلوکز و عصاره مخمر شد.

نتایج: اثر چندین منبع کربن و نیتروژن بر روی بیومس، تولید لیپید و اسیدهای چرب بررسی شد. بیشترین میزان تولید لیپید در محیط کشت حاوی لاکتوز و عصاره مخمر یافت شد (۲۶/۶۶درصد). لینولئیک اسید تنها در حضور لاکتوز و عصاره مخمر (یا پپتون) تولید شد (۲۵/۷درصد). با این حال، *مورتیرلا وینشیا* بیشترین سطح لینولئیک اسید (۵۲/۷۶درصد) را در محیط کشت حاوی پپتون تولید کرد. لینولنیک اسید تنها در حضور لاکتوز و تریپتون به دست آمد.

بحث و نتیجه‌گیری: در این مطالعه، لاکتوز به عنوان منبع کربن بیشترین تأثیر را بر تولید لیپید داشت. علاوه بر این، لینولئیک اسید در حضور لاکتوز تولید شد، بنابراین لاکتوز به عنوان بهترین منبع کربن انتخاب شد. پپتون و تریپتون به عنوان منبع نیتروژن به ترتیب برای تولید لینولئیک و لینولنیک اسید توسط *مورتیرلا وینشیا* انتخاب شدند. تمام این یافته‌ها نشان می دهد که سویه مورتیرلا یک کاندید بالقوه برای افزایش محتوای لینولئیک اسید و لینولنیک اسید است. همچنین، این شرایط ساده محیط کشت می‌تواند برای تولید لینولئیک اسید و لینولنیک اسید در مقیاس بالاتر، برای اهداف صنعتی استفاده شود.

واژه‌های کلیدی: اسید چرب، استخراج لیپید، کروماتوگرافی گازی، *مورتیرلا وینشیا*

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