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Temperature Optimization for Arachidonic and Eicosapentanoic Acids Production by Oomycete *Pythium irregulare* LX

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Abstract: The biomass, substrate utilization, lipids and individual Poly Unsaturated Fatty Acid (PUFA) profiles were studied in oomycete *Pythium irregulare* LX and isolated in Azerbaijan National Academy of Sciences (AMEA), during the fermentation process at different temperatures. The results were then applied to determine an optimized incubation temperature mode for target PUFA production. When temperature did not shift, the optimal lipid, EPA and AA production occurred at the incubation temperature of 25°C when the temperature shifting technique was implemented, greater yields occurred for cultures incubated for 5 days at 28°C followed by 2 days at 15°C.

Key words: Temperature optimization, *Pythium irregulare* LX, Poly unsaturated fatty acid, AMEA, Iran

INTRODUCTION

Poly unsaturated fatty acid involve the fatty acid with two or up double bond. Linoleic acid (C18:2n6c), α-linolenic acid (C18:3n3), γ-linolenic acid (C18:3n6), dihomo-γ-linolenic acid (20:3n6), arachidonic acid (C20:4n6), eicosapentanoic (C20:5n3) and docosahexaenoic acid (C22:6n3) are the main fatty acids. These fatty acids are found in animals and plants but there are high variety of fatty acids in microorganisms such as alga, fungi and bacteria (Amate *et al*., 2002; Averina and Kutyrev, 2011; Bligh and Dyer, 1959). The fatty acids in microorganisms are found storage fats and membrane phospholipids. The main sources of 18 carbon unsaturated fatty acids are plants seed (Brown *et al*., 2010; Cheng *et al*., 1999). Arachidonic acids extracted from animals particularly the liver of pig and eicosapentanoic acid docosahexaenoic acid extracted from the oil of fish. Unsaturated fatty acid rate is very low in agriculture products and it depends on climate condition. Eicosapentanoic acid and docosahexaenoic acid obtained from fish oil have palatable odor and flavor but because of attendance of more among of cholesterols and a little indissoluble compositions in it, it is necessary find of new resources for these acids. In microorganisms one of the important of factors for unsaturated fatty acids biosynthesis is incubation temperature. The researches have shown that low temperatures have positive effects in unsaturated fatty acids biosynthesis. In present research effect of temperature on lipogen *Pythium irregulare* LX oomycete is investigated and optimized.

MATERIALS AND METHODS

Medium and culturing condition: The Lipogen micromycetes *Pythium irregulare* LX used during present investigation was isolated in Institute of Microbiology, National Academy of Science of Azerbaijan, Baku. The medium for oomycetes cultivation was composed of 2% glucose, 0.5% yeast extract (Fisher scientific) and 0.1% KH_2PO_4 (pH = 6.2). Microorganism was incubated at three difference temperature 15, 21 and 28°C on a rotary shaker (200 rpm) in aerobic condition and deep culturing.

Measurements of reducing sugar and DO: Reducing sugar rate were measured by Somogyi-Nelson Method and Dissolved Oxygen (DO) measured by INGOLD Polar Graphic O_2 sensor (with diameter 12 mm and 100 nm limited and accuracy 30 nm).

Separation of lipid compositions: For lipids extraction Floch and Bligh-Dyer Methods were used (Folch *et al*. (1957) and Khozin-Goldberg *et al*. (2011). Lipids fatty acid composition was determined by Highly effective Liquid Chromatography (HPLC). Methylation lipids as well as lipid methanolysis mix were separated by liquid chromatography with ultraviolet detector ((l = 250 nm) KOBO marked). The structure of fatty acids was determined by mass-spectrum and iodine number was determined by ГОСТ 5475-69 (Kishimoto *et al*., 2003). The calculated date was done in meaningful level $p<0.05$.

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RESULTS AND DISCUSSION

Growth mechanism in oomycete is different from other mono cellular microorganisms. All of the cells in poly cellular filamentous fungi within growth process have role in growth and divergence of hypha. The results extracted from growth and lipogenesis in *Pythium irregulare* LX in three different temperature present specific characteristics compared with other fungi. Growth curve in 15, 21 and 28°C is formed in 5, 6 and 7 days, respectively that have shown in Fig. 1.

Stationary phase is very short and growth curves show rapid downfall of maximum biomass at short time that it can be due to lyses of biomass and/or oxygen shortage in follicle. The quantity of biomass had a little difference, in days 5, 6 and 7 at temperature 28, 21 and 15°C was 10.1, 9.71 and 8.9 g L^{-1}, respectively. The temperature not only effects on growth as well as effects on other metabolic activities, for example substrate consumption and pH changes. Substrate consumption in higher temperatures is more. The results of reducing sugar exist in broth medium in fermentation cycle have shown in Fig. 1. In every temperatures the change of reducing sugar rate are conjunction with biomass aggregation. The maximum utilization of carbon in 28, 21 and 15°C was in days 4, 6 and 9. In these times 92.5-95% glucose were used. The changes of pH were usual for fungi cultures. In early growth phases, acidity of medium decreased in result of ammonium decrease and aerobic consumption of carbon source due to production of carbonic acids which it has presented in Fig. 2.

In higher temperatures in early stages, the rate of pH in conjunction with metabolism rapidity decreased sharply. In further stage, the rate of pH because of biomass lyses, ending and/or suddenly decrease carbohydrate in medium. In Fig. 2 also dissolved oxygen in medium has shown in stationary phase in high temperature. In room temperature 7.52 mg oxygen solves in 1 L water. Therefore, there will be enough oxygen on mycelium surface within fermentation. Despite this topic when the mycelium definite measure constitute oxygen limit. The energy supply in lipid and cell membrane composition involve both primary and secondary metabolites. The lipid percents in biomass and lipids rates in per every liter in trial three different temperatures have shown in Table 1.

As Table 1 shows the biomass amount has increased in active growth period. In 28 and 21°C lipid product continued another 1 day after maximum biomass amount and then in that temperatures were stable in level 28-29 and 19-19.7%, respectively. In 15°C lipid rate and maximum biomass had overlap and was stable in level 19.5% for 2 days. Despite the lipid of biomass and stability compared with medium volume in 21 and 15°C these amount were very close-set, about 1.75 and 1.74 g, respectively. Lipid rate per every liter medium approached to 2.88 g in 28°C. It should have mentioned it is needed to say that the results of this presentation had not overlap with other researches. For example some researchers have attempted to show that the lipid amounts of biomass increase in temperature lower than optimal growth temperature (11, 12 and 13) (Fig. 3). Whereas, in present research, the lipid rates of biomass were higher in 28°C

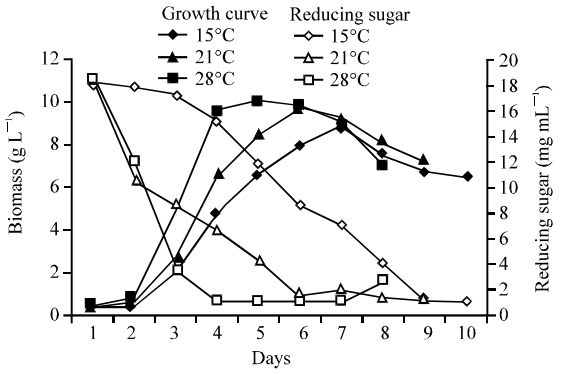

Fig. 1: Growth curve in three different temperature 15, 21 and 28°C in *Pythium irregulare* LX and the rate of consumption of medium reducing sugar

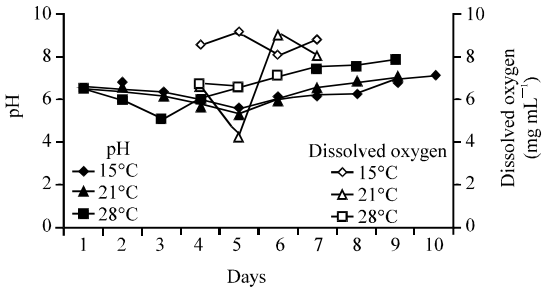

Fig. 2: The changes of Dissolved Oxygen (DO) and medium acidity in *Pythium irregulare* LX in temperature 28, 21 and 15°C within growth time

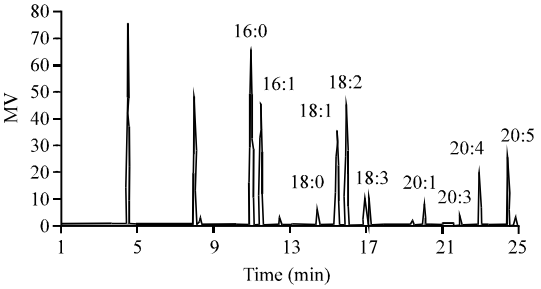

Fig. 3: Fatty acids chromotogram in *Pythium irregulare* LX in temperature 28°C

Table 1: Lipid biosynthesis in *Pythium irregular* LX in temperature 28, 21 and 15°C

		Incubation time (day)									
Extract rate	Temperature (°C)	1	2	3	4	5	6	7	8	9	10
Lipid rate in biomass (%)	28	7.0	12.0	20.0	25.0	28.5	29.0	28.4	27.0	-	-
	21	7.0	8.0	10.4	12.2	15.5	19.2	19.7	19.6	19.0	-
	15	7.0	7.5	7.5	13.6	16.0	19.5	19.5	19.5	18.7	18
Lipid extract rate (mg L^{-1})	28	28.0	99.6	1000.0	2400.0	2880.0	2860.0	2582.0	1912.0	-	-
	21	25.2	41.3	218.0	680.0	1035.0	1505.0	1786.0	1584.0	1412.0	-
	15	25.2	27.0	193.0	653.0	1064.0	1546.0	1735.0	1463.0	1255.0	1166

Table 2: Biosynthesis of eichisapentane acid and arachidonic acid in *Pythium irregular* LX in temperature 28, 21 and 15°C

		Incubation time (day)									
Extract rate	Temperature (°C)	1	2	3	4	5	6	7	8	9	10
Eichisapentane acid	28	10.0	50.00	80.00	100.0	120.0	90.0	100.0	27.0	-	-
extract rate (mg L^{-1})	21	10.0	18.00	10.40	16.0	22.0	24.0	15.0	3.0	3.0	-
	15	12.0	18.00	15.00	19.0	20.0	21.0	11.0	5.0	4.0	2.0
Arachidonic acid	28	10.0	30.00	50.00	65.0	73.0	70.0	80.0	68.0	-	-
extract rate (mg L^{-1})	21	8.0	12.00	9.00	10.0	13.0	15.0	8.0	4.0	3.0	-
	15	2.0	0.02	0.02	5.0	6.0	9.0	4.0	3.0	4.0	3.0

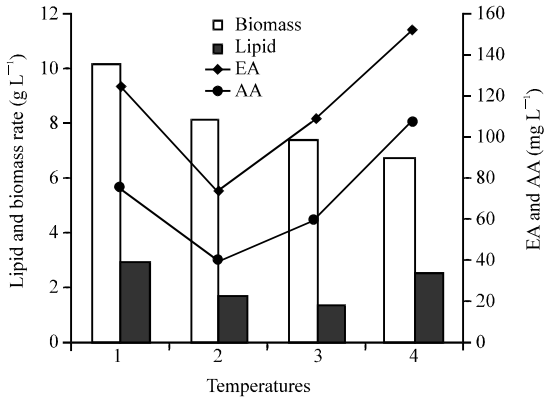

Fig. 4: The rate of biomass, lipid Eicosapentanoic Acid (EA) and Aarachidonic Acid (AA) by temperature change in *Pythium irregulare* LX; 1) 6 days in 28°C ; 2) 3 days in 28°C + 4 days in 15°C; 3) 4 days in 28°C + 3 days in 15°C and 4) 5 days in 28°C + 2 days in 15°C

than two other temperatures. By results of HPLC, the fatty acids of lipids in *Pythium irregulare* LX consist of myristic acid, myristoleic acid, palmitic acid, heptadecane acid, oleic acid, arachidonic acid and cis 5, 8, 11, 14 and 17 eicosapentanoic acid.

In chromatogram a little amount of estaeric, cis 11 eicosanoic and cis 8, 11 and 14 eicosatrienoic acid also were observed. The results of analysis of the main fatty acids in this study, eicosapentanoic acid and arachidonic acid in temperatures 15, 18 and 21°C in the course of incubations have shown in Table 2. The maximum amount of eicosapentanoic acid was collected in 28°C at 5th day and arachidonic acid amount reach to 73 mg L^{-1}. The maximum amount of eicosapentanoic acid (80 mg L^{-1}) was collected in 7th day which in this time eicosapentanoic acid rate decreased to 100 mg L^{-1}. The peak of eicosapentanoic and arachidonic acid product in 21, 15°C were in 6th day. Despite this topic, more important lipids and main individual fatty acids rates were lower than temperature 28°C. Eicosapentanoic and arachidonic acid rates in 21°C were 24 and 15 mg L^{-1}, respectively and in 15°C were 21 and 9 mg L^{-1}, respectively. Whole individual fatty acids amount decreased rapidly every three temperatures after reach to maximum rate. The reason of this affair is not distinct but probably the leak of intracellular fatty acids to out of cell and/or neutral fatty acid exchange to free fatty acid have role. Therefore, according to outcome of experience the maximum products of arachidonic acid and eicosapentanoic acid in 15 and 21 were in day 6 and in 28°C were in 5th day.

In subsequent stages of research is used from temperature changes method for increase of arachidonic acid and eicosapentanoic acid amount in the mentioned culture. The effect of temperature on product of biomass, lipid, arachidonic acid and eicosapentanoic acid investigated with three methods. The cultures were grown in temperature 25°C for 3-5 days then incubation temperature were decreased to 15°C and biomass were collected in 7th day. The biomass, lipid and unsaturated fatty acids rates of taken samples within growth are shown in Fig. 4. Increase in lipid product is not show in 28°C for 3 days and fallow it 4 days in 15°C within incubation and lipid amount in biomass kept constant in level 20%. It seems that in stage switching nitrogen rate quantity was enough in medium. The biomass amount decreased in 28°C with change within incubation duration from 3-5 days, lysis can be observed in 5th days but lipids product rate rose in proportion to medium volume. In this option, the biomass lipid rate within 5 growth days in 28°C was 28.5% and lipid product in proportion to medium

volume decrease to 288 mg L^{-1} in 15°C in next 2 growth days. But lipid product compare with first option (in 28°C for 4 days and then 3 days in 15°C) decreased nearly 2 times. Product increase of eicosapentanoic acid and arachidonic acid caused increase of lipid product. The quantity of eicosapentanoic acid in 28°C with 5 days and in 15°C with 2 days incubation with 21% increase reached to 152 mg L^{-1} and the quantity of arachidonic acid with 32% increase in same conditions reached to 108 mg L^{-1}. Therefore, the optimum temperature for product of arachidonic acid, eicosapentanoic acid and lipid in fix temperature was 28°C and in temperature change method incubation for 5 days in 28°C as well as incubation for 2 days in 15°C was optimum temperature.

CONCLUSION

It can be concluded that the results of biomass, substrate utilization, lipids and individual PUFA profiles were studied in *Pythium irregulare* during the fermentation process at different temperatures showed when temperature was not shifted, the optimal lipid, the optimal lipid, EPA and AA production occurred at the incubation temperature of 25°C when the temperature shifting technique was implemented, greater yields occurred for cultures incubated for 5 days at 28°C followed by 2 days at 15°C.

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