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Gamma-Linolenic Acid Production with Simultaneous Lipid-Content Reduction in Soybean Meal after Solid-State Fermentation

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Abstract: The aim of this study was to evaluate changes in fatty acids composition, following the production of gamma-linolenic acid and the reduction of lipid concentration after agro-industrial substrates fermentation. A *Mucor circinelloides* strains was selected among seven fungal strains as the most effective gamma-linolenic acid producer in submerged cultivation. This filamentous fungus had the action lipolytic during solid-state fermentation with agro-industrial substrates, followed of the production of 3.0 g/kg BP of gamma-linolenic acid in cultures with soybean meal. Canola and sesame oil mixture (2% w/w) provided a gamma linolenic acid production of 6.2 g/kg BP. Moisture of the substrate created a compact mass preventing fungus utilized the substrate completely. When rice husks was added to the substrate, supplemented with canola plus sesame oils 2%, it was observed the gamma-linolenic acid production of 8.4 g/kg BP and total lipid was reduced 3.3-fold. These results represent a new perspectives and promising for the oleaginous fungi in solid-state fermentation cultivation process, objectifying the production of feed with improved nutritional quality and at low cost.

Keywords: Agro-industrial substrate, fatty acid, fermentation, filamentous fungi, lipid, *Mucor circinelloides*

INTRODUCTION

Filamentous fungi are considered the most important polyunsaturated Fatty Acid (PUFA) producers in submerged or solid-state fermentation. Solid-State Fermentation (SSF) is a process in which fungi grow on a moist solid substrate in the absence of free water (Hölker and Lenz, 2005) and it is accepted as the most economical process for the production of enzymes, oil and spores used in biocontrol (Rahardjo *et al*., 2005; Jang and Yang, 2008). Agro-industrial wastes, e.g., soybean meal, sugarcane bagasse, cassava husks, wheat bran and citric pulp, are available in large quantities, are renewable and inexpensive and often contain adequate levels of carbohydrates, proteins, oils and minerals to allow their use for feed formulation (Gema *et al*., 2002; Allan and Rowland, 2005).

Agro-industrial substrates, such as soybean meal, have high oil concentrations, rendering inadequate to be used for feed, because the lipids metabolism as energy sources changes with the different dietary habits of animals (Romarheim *et al*., 2007); furthermore, these lipids may accumulate in the adipose tissue. The oil extraction with hexane is commonly used in cereals applied in the feed formulation's; it is considered an expensive process that causes the loss of essential amino acids and the nutrients` suppression from substrates resulting in an increase in animal mortality rate (Sharma *et al*., 2002). Lipolytic metabolism observed in some filamentous fungi and yeasts strains growing in agro-industrial substrates to yield gamma-linolenic acid (GLA) can be an alternative in the defattening process applied on substrates for feed formulation (Abu *et al*., 2000; Yano *et al*., 2008).

Filamentous fungi can accumulate large quantities of lipids comprising a significant fraction of GLA. This fatty acid has important roles in the structure and function of biological membranes and it is a precursor of many biologically active compounds (Kenny *et al*., 2001; Charalampopoulos *et al*., 2002). *Mucor* genus has been considered an oleaginous fungus producing high levels of GLA in controlled culture conditions (Dyal *et al*., 2005; Tauk-Tornisielo *et al*., 2007; Tauk-Tornisielo *et al*., 2009). The main target of this study was to evaluate changes in fatty acid composition, following the production of GLA and the reduction of lipid concentration in the agro-industrial substrates cultivated by *M. circinelloides*. The effect of soluble carbon supplements (carbohydrates and/or vegetable oils) and rice husks as inert supports for lipid reduction and GLA production were also investigated. This work is a preliminary study of lipid reduction and GLA production

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by oleaginous fungi using agro-industrial substrates for further feed formulation.

MATERIALS AND METHODS

Microorganisms and growth conditions: The experiments were performed in the period 2009 to 2010 in the Microbiology laboratory of Environmental Studies Center Collection, CEA, São Paulo State University, UNESP, Brazil. *Mucor circinelloides* 122 (isolated from Cerrado area soil), *M. circinelloides* 31 (isolated from Caatinga area soil), *M. circinelloides* J17, *Circinella simplex* J2, *C. simplex* J3, *Mucor hiemalis* and *Mucor* sp. (isolated from Atlantic Forest soil) are stored in the Environmental Studies Center Collection, CEA/UNESP, Brazil. The microorganisms were maintained on oat-agar medium at 4ºC and cultured periodically. The cultures were inoculated in the same medium and incubated for spore production during five days at 28ºC. The spores were harvested and suspended in sterile saline solution. The suspension concentration was adjusted to 10^7 spores/mL, using for liquid and solid media inoculation. Submerged cultivation: Liquid cultures were prepared in Vogel's salt solution (Vogel, 1956) supplemented with 10 g/L of glucose and yeast extract, pH 5.8. Erlenmeyer flasks (250 mL) containing 50 mL of medium were inoculated with 5 mL of the spore suspension and incubated in a rotary shaker at 150 rpm for 96 h at 25ºC.

Solid-state fermentation: Wheat bran, soybean meal, cassava husks, citric pulp and sugarcane bagasse were evaluated. Five grams of each dry substrate were used for cultivations in Erlenmeyer flasks (250 mL) and initial moisture was 50% (v/w) by the addition of distilled water. Cultivation with citric pulp, cassava husks and sugarcane bagasse substrates were supplemented with 1% (w/w) yeast extract to facilitate fungal growth. The autoclaved substrates were inoculated with 1 mL of spores in suspension and incubated for 168 h at 25 ºC.

Carbon supplementation and effect of rice husks addition: Soybean meal was supplemented with 2% (w/w) vegetable oil (canola, sunflower, or sesame) or soluble carbohydrate (glucose or maltose). Other supplements were prepared with 2-4% (w/w) of glucose, canola, or sesame oil. In other experiments, rice husks at variable proportions (3:1, 3:2 or 3:3 w/w) were added to soybean meal supplemented or not with canola and sesame oils at 2% (w/w) concentration. All substrates were homogenized, sterilized, inoculated and incubated for 144 h at 25ºC.

Lipid extraction and fatty acid analysis: Fermented substrates were dried at 65ºC until their weights stabilized. The lipid fractions were extracted according to Folch *et al*. (1957). The lipid fraction was esterified according to Morrison and Smith (1964). The upper phase, containing the fatty acids methyl esters, was analyzed in a gas chromatograph, equipped with a FFAP megabore column (30 m×0.1 mm) and a FID detector; hydrogen was the carrier gas. The analyses were run from 50 to 220ºC, with the injector at 210ºC and the detector at 250ºC. The fatty acid was identified by reference to verified standards (Sigma-Aldrich).

Table 1: Biomass, total lipid content and gamma linolenic acid production by filamentous fungi

Strains	Biomass (g/L)	Total lipid (g/L)	GLA (mg/L)
M. circinelloides J17	8.6±0.3	0.40±0.01	10.2±1.2
M. circinelloides 31	7.2±0.4	0.29±0.01	1.6±0.1
M. circinelloides 122	4.1±0.2	0.19±0.01	19.9±1.7
C. simplex J2	5.6±0.1	0.55±0.02	12.8±0.9
C. simplex J3	5.7±0.4	0.62±0.04	14.5±1.1
M. hiemalis	8.0±0.2	0.35±0.02	10.1±0.9
Mucor sp.	7.8±0.3	0.49±0.03	13.1±0.8

Statistical analysis: Experiments were carried out in triplicate and most important experimental data was subjected to analysis of variance and Tukey's test ($p = 0.05$) using the Statistical Analysis System (SAS Institute, 2002).

RESULTS AND DISCUSSION

Selection of strains: Initially, the biomass, total lipid content and GLA production by seven fungal strains was assayed after 96 h of submerged cultivation (Table 1). *C. simplex* J2 and J3 presented the same biomass, but different concentration of total lipid and GLA. Among the *Mucor* spp. strains, there was no correlation between biomass production, lipid accumulation and PUFA production. *M. circinelloides* 122 produced the highest GLA concentrations, followed the lowest biomass and total lipid production (4.1and 0.19 g/L, respectively). These results can be related to the physiology of the strains within their natural environmental conditions, presenting differences between lipid accumulation and GLA production without any correlation among themselves (Fakas *et al*., 2006; Tauk-Tornisielo *et al*., 2007). The higher GLA production observed by *M. circinelloides* 122 led us to choose this strain for the following studies.

Agro-industrial substrates for fermentation: *M. circinelloides* 122 was cultivated in SSF with different agro-industrial substrates for 168 h, namely Bioproduct (BP). Initial lipid content and fatty acids profile of the substrates were previously characterized and are shown in Fig. 1 as time zero. Fungal growth was observed in all substrates, except sugarcane bagasse and citric pulp

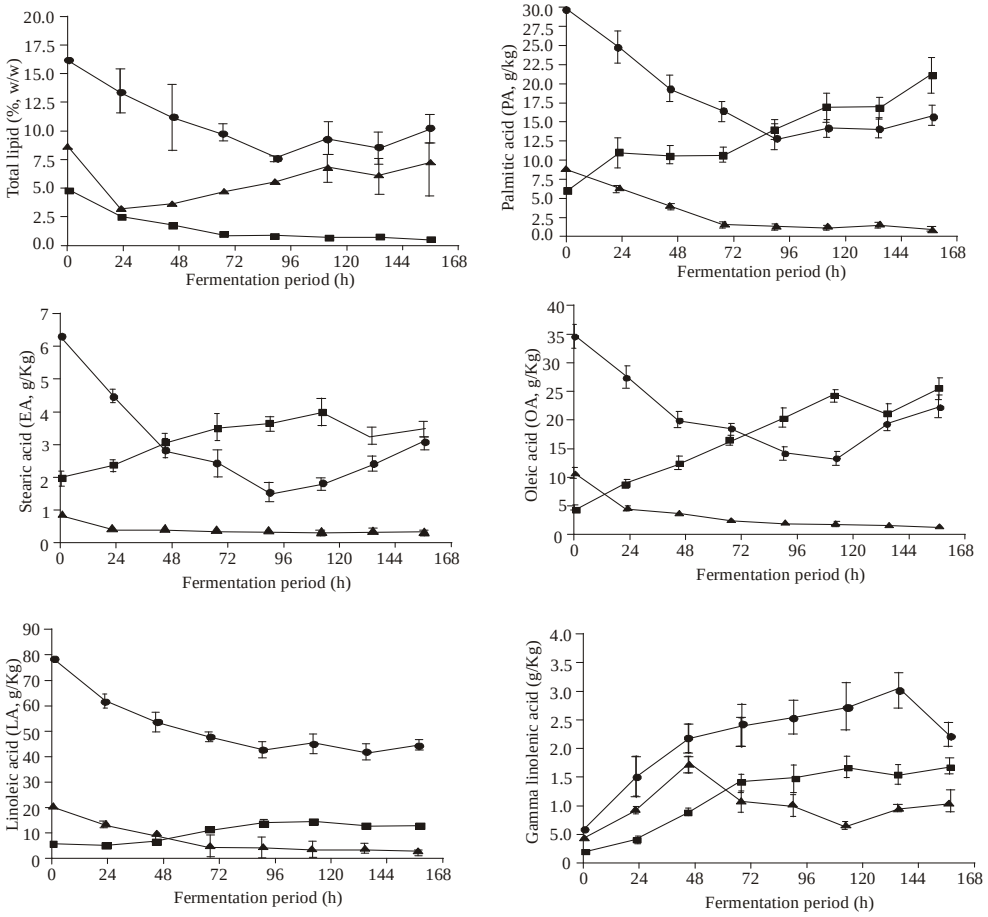

Fig. 1: Total lipid content (a), saturated fatty acids (b, c) and polyunsaturated fatty acids (d, e, f) after solid-state fermentation with (■)cassava peel, (●) soybean meal and (▲) wheat bran for 168 h at 25ºC.

during the cultivation period. Fungal growth was observed in cassava peel supplemented with yeast extract after 48 h, followed by an increase in total lipid accumulation during the cultivation period. The lipolytic metabolism of *M. circinelloides* 122 was observed with soybean meal and wheat bran reducing total lipid content for 36.9% and 89.1%, respectively (Fig. 1a). The concentration of Saturated Fatty Acids (SFA), such as palmitic and stearic acid, in soybean meal and wheat bran decreased for 51.6 and 10.0%, respectively (Fig. 1b and 1c). A decrease of the PUFA concentrations in soybean meal and wheat bran was also observed. On the other hand, there was an increase of the GLA concentrations, reaching maximum values at 144 h in soybean meal (3.0±0.3 g/Kg BP) and at 48 h in wheat bran (1.7±0.7 g/Kg BP). *M. circinelloides* cultures in cassava peel, although had presented a decrease in final total lipids, there was an increase of all fatty acids during fermentation, including gamma-linolenic acid. Abu *et al*. (2000) reported that microorganisms can use the lipids from the substrates to convert in microbial component and PUFA, being incorporated into the cell membrane by enzymatic complex. Aggelis and Sourdis (1997) mentioned that either fatty acids are degraded for growth requirements or used as a substrates for the endocellular biotransformation process, leading to concentration changes and production of 'new' fatty acids, that were not previously present in the substrate.

Recently, these microorganisms were used as a tool to reduce the lipid content in fish meal used as animal feed (Yano *et al*., 2008). Soybean meal was selected for further studies by present highest GLA production followed reduction of total lipid content. Thereafter, it an excellent protein and energy source and is widely applied by most companies in the formulation of the feed for poultry, fish and swine (Mathis *et al*., 1999).

Supplementation of the substrates: The supplementation of the substrates was based on the hypothesis that the addition of carbohydrates or vegetable oils may stimulate fungal growth and the maintenance of the organism; yet, may allow the organism to convert unsaturated fatty acid, e.g. oleic acid and linoleic acid, to GLA. Initial lipid concentrations, SFA, PUFA and GLA

Table 2: Total lipid and fatty acid composition of soybean meal supplemented with rice husks and canola and sesame oils 2% after solid-state fermentation

Rice husks		TL (%, w/w)	SFA (%, w/w TL)	PUFA (%, w/w TL)	GLA (%, w/w TL)	GLA (g/Kg BP)
Without oil						
3:1	Control	14.1±1.0	28.0±3.6	58.4±5.4	ND	ND
	144 h	5.0±1.3	13.5±5.8	78.5±4.8	6.0±3.4	3.0±0.4
3:2	Control	15.8±2.0	23.4±6.1	62.3±7.8	ND	ND
	144 h	5.0±3.1	13.8±4.3	79.2±6.6	8.8±2.5	4.4±0.5
3:3	Control	16.2±1.9	21.6±6.2	63.5±5.8	ND	ND
	144 h	3.4±2.1	16.2±4.2	74.7±3.4	5.2±4.1	2.7±0.4
With oil						
3:1	Control	15.8±1.2	30.4±5.3	59.8±3.6	ND	ND
	144 h	7.5±2.4	11.4±2.6	83.6±3.9	7.1±3.2	5.3±0.6
3:2	Control	17.4±3.4	23.0±3.7	65.2±5.2	ND	ND
	144 h	5.2±2.3	9.7±3.0	84.1±6.3	16.1±3.6	8.4±0.9
3:3	Control	16.8±4.2	21.8±4.3	64.8±6.1	ND	ND
	144 h	2.7±2.0	12.9±3.3	79.1±4.9	17.0±4,1	7.6±0.8

TL: total lipid; SFA: saturated fatty acids (palmitc acid, stearic acid); PUFA: polyunsaturated fatty acids (oleic acid, linoleic acid, GLA)

Table 3: Total lipid and fatty acid composition of soybean meal after solid-state fermentantion

Supplements		TL (%, w/w)	SFA (%, w/w TL)	PUFA (%, w/w TL)	GLA (%, w/w TL)	GLA g/Kg BP
Glucose	Control	14.3±3.2	21.2±4.2	69.7±6.3	0.4±2.1	0.6±0.0
	144 h	8.1±0.8	16.4±3.6	78.9±5.2	5.5±2.1	4.5±0.9
Maltose	Control	14.9±5.6	19.9±5.8	68.3±4.6	0.3±1.2	0.4±0.0
	144 h	9.2±0.4	14.4±6.1	79.6±6.1	3.3±3.1	3.0±0.2
Canola oil	Control	16.1±2.1	32.8±5.2	54.0±3.3	ND	ND
	144 h	7.0±0.7	10.9±4.4	82.9±5.1	6.6±3.2	4.6±0.5
Sunflower oil	Control	16.7±3.1	33.3±6.2	55.0±5.4	ND	ND
	144 h	6.6±0.6	12.0±4.2	83.6±4.8	5.9±4.2	3.9±0.4
Sesame oil	Control	15.9±4.1	20.9±2.7	68.1±6.6	ND	ND
	144 h	8.4±0.4	11.3±3.6	82.3±5.8	6.5±3.1	5.5±0.3
CS 2%	Control	17.1±3.3	31.2±5.3	78.6±7.8	ND	ND
	144 h	9.7±4.1	11.9±3.6	83.8±3.4	6.4±3.2	6.2±0.3
CS 4%	Control	19.8±4.2	28.7±4.3	56.9±4.8	ND	ND
	144 h	11.5±5.2	23.6±5.6	69.7±5.6	4.8±2.4	5.5±0.6
CGlc 4%	Control	16.8±5.9	30.5±4.1	58.9±3.6	ND	ND
	144 h	9.1±4.6	12.4±5.8	82.3±3.6	5.3±1.2	4.8±0.5
SGlc 4%	Control	17.0±6.0	21.6±3.7	64.4±3.9	ND	ND
	144 h	8.4±3.1	19.9±4.9	73.2±5.2	4.8±2.5	4.0±0.2

TL: total lipid; SFA: saturated fatty acids (palmitc acid, stearic acid); PUFA: polyunsaturated fatty acids (oleic acid, linoleic acid, GLA); CS: canola plus sesame oil; CGlc: canola oil plus glucose; SGlc: sesame oil plus glucose

of the substrates and bioproducts are depicted in Table 2. Linoleic acid was dominant fatty acid in all substrates, followed oleic acid and palmitic acid, respectively.

Lipid content in bioproducts decreased to 39.5-61.7% after fermentation. Interestingly, SFA decreased in all bioproducts; but in counterpart there was an increased of PUFA at final fermentation of approximately 50% in canola oil and sesame oil, respectively. GLA concentrations in the bioproducts also increased in the cultures with sole carbon supplements, except in the cultures with maltose which there was no effect in GLA production (3.0 g/kg BP). The experiments with sesame oil, canola oil and glucose as sole carbon supplements presented the highest values of GLA (5.5 g/kg BP, 4.6 g/kg and 4.5 g/kg BP, respectively).

Mixtures of canola and sesame oils (CS 2%) provided a GLA production of 6.2 g/Kg BP which was higher than CS 4% (5.5 g/kg BP), representing 6.4 and 4.8% of GLA in total lipids. Mixtures of oil plus glucose 4% provided values lower of GLA production (4.8 and 4.0 g/kg BP, respectively). These results are according with Stredansky *et al.* (2000) and Jang *et al*. (2000) which various substrates were supplements with vegetable oils providing an increase in GLA production.

Effect of the addition of rice husks to the substrate: In previous experiments it was observed that the moisture of the medium, required for fungal growth, created a compact mass which may have interfered with microbial respiration, which can affect negatively the substrate utilization and fermentation processes. Rice Husks (RH) consist, mainly, of crystallized or amorphous cellulose, lignocelluloses and silica, forming a complex structure that is difficult to be used for fungal growth; however can be used to prevent fermentation-mass packing. The effect of RH on fermentation was verified by adding various amounts to soybean meal, supplemented or not whit vegetable oil (Table 2).

Addition of the RH to the soybean meal increased the lipid consumption by the fungus which achieved 2.7% in RH 3:3 supplemented with 2% canola oil plus sesame oil. SFA also decreased significantly in the bioproducts with

the better results in RH 3:2 supplemented with 2% canola oil plus sesame oil (9.7% w/w TL). There was no significant increase of PUFA concentration in total lipid in these conditions of fermentation compared with soybean meal supplemented with vegetable oil (Table 3); however GLA concentration in the bioproduct increased 35.5% (8.4 g/kg BP) when *M. circinelloides* was used to grow in RH 3:2 with 2% of canola oil plus sesame oil, representing 16.1% in the total lipids. Cultivations in soybean meal with RH without supplementation reduced total lipid and SFA together with increase of PUFA in the bioproducts, but GLA productions was not increased in these conditions of fermentation, suggesting that the supplementation with vegetable was essential for GLA accumulation in the bioproducts. The addition of inert supports to the substrate of fermentation has been studied in the literature. Certik *at al*. (2006) reported that the ratio of inert support added to substrate seems to be dependent of the fungal strain, nutrient availability and the substrate composition. Gema *et al*. (2002) and Certik and Adamechova (2009) observed that the addition of a material to increase substrate porosity increased GLA production which improved fungal growth and lipid accumulation due to the activities of Δ^9-*desaturase* and Δ^6-*desaturase* that promote the unsaturation of linoleic into GLA.

These are the primary results of microbial cultivation in soybean meal for GLA production with simultaneous total lipid reduction. Thereafter, other parameters involved in these fermentations process should be performed to evaluate the use of this process for animal feed formulations.

CONCLUSION

Mucor circinelloides 122 was selected as the most promising strain among seven strain cultivated in submerged fermentation. *M. circinelloides* cultivated in agro-industrial substrates presented an important potential for total lipids reduction and increasing gamma-linolenic acid concentration. Supplementation with 2% canola oil and sesame oil and the addition of rice husks 3:2 decreased total lipids in 70.1% and saturated fatty acids in 57.8% in the bioproducts. Polyunsaturated fatty acids increased 29% in total lipid and an important increase in the GLA production of 35% was observed in these conditions. The lipolytic metabolism of this strain was important since the soybean meal used in feed formulations is defatted by use of organic solvents. *M. circinelloides* cultivation in solid-state fermentation with lipolytic metabolisms represents a new and promising process for feed production with improved nutritional quality and at low cost. Further studies are required to investigate the physical-chemical parameters of cultivation necessary for industrial scale-up of the process and crude protein and essential amino acids composition of the substrates.

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