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Characterization of Microbial Lipid Production with *Mucor rouxii* on Pure Carbon Substrates

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A B S T R A C T

Oleaginous mould *Mucor rouxii* was cultivated in the medium containing glucose, starch and cellulose with an initial C/N ratio of 60. The biochemical behaviors of *Mucor rouxii* were examined: The highest lipid yield (4.9 g/L) was found with glucose as the carbon substrate. Starch was good for biomass production (15.5 g biomass/L medium). The Lipid content in biomass with starch as the carbon substrate was less than glucose (25%). The maximum lipid yield was increased (up to 5.8 g/L) with increasing the starch concentration to 60 g/L. Cellulose did not support lipid production. Significant quantities of α-amylase (0.5 and 1.2 IU/mL) and cellulase (0.19 IU/mL) were produced. The research suggest that in order to consume complex carbon substrates oleaginous mold should secrete complex enzymes to break down the substrates into simpler sugars and channelize them for lipid production. This study is one of the first in utilizing cellulose as a carbon source for lipid and Gamma Linoleic Acid (GLA) accumulation. The content of GLA varied considerably with the substrates.

Introduction

M. rouxii was cultivated with three pure carbon sources (glucose, starch and cellulose) to compare its lipid production and physiological responses between potato processing wastewater and pure carbon sources. It has been demonstrated in the literatures that, oleaginous microorganisms (yeasts and fungi) exhibit various characteristics in both lipid yields and lipids' fatty acid composition when cultivated with difference carbon substrates despite that these carbon substrates are biochemically similar (Papanikolaou *et al.*, 2007). For instance, *Mortierella isabelliana* and *Cunninghamella echniulata*, two oleaginous fungi cultivated on glucose, starch, pectin and lactose based media, showed different biomass production. Glucose and starch was suitable for biomass growth of the two fungi; lactose favoured biomass production of *M. isabelliana* but did not support the growth of *C. echniulata*. Both fungi produced more lipids with glucose as the carbon substrate than with starch. Pectin was an inadequate substrate for biomass growth and lipid production for *C. echniulata*, but it supported the growth of *M. isabelliana* and lipid production. Cellulose is the most abundant organic carbon source in the nature. However, there are very limited studies on direct fermentation of cellulose into microbial

lipids by oleaginous microorganisms. Starch is another abundant carbon source. In this chapter, physiological responses of oleaginous fungi such as, biomass production, substrate uptake, secretion of hydrolytic enzymes, and lipid accumulation with cellulose or starch as the organic carbon substrate were studied. Glucose, one of the simplest sugars, was also studied as a comparison study. *M. rouxii*, a known lipid and GLA producer (Ahmed *et al.,* 2006), was used in this study.

Materials and Methods

Microorganism and cultural conditions

Mucaraceous fungi *Mucor rouxii* DSM1191 was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The culture was stored in the laboratory on potato dextrose agar slants at 4^{o}C. The lipid production medium consisted of three groups of components: i) mineral salts containing (g/L), $CaCl_2$, 0.1; KH_2PO_4, 2.5; $FeSO_4$, 0.02; NH_4Cl, 0.01; $MgSO_4$, 0.5; $MnSO_4$, 0.003; and $CuSO_4$, 0.002; ii) nitrogen source of 0.5 g/L $(NH_4)_2SO_4$; and iii) carbon source. Three types of carbon sources were examined and they were glucose, starch and cellulose (Sigma Aldrich, Ireland): glucose and cellulose concentrations tested were 30 g/L and two starch concentrations were studied, 30 and 60 g/L.

Inoculation of the fungal culture was performed as follows: 1.0 g of mycelia were taken from potato dextrose agar plates and cultured in yeast extract malt extract agar (YM agar) broth containing 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, and 3 g/L malt extract (pH of the medium was 5.5) for 48 h in a shaker incubator at 30±1ºC at 180 rpm. After 48 h the mycelium was harvested and homogenized using sterile glass beads (0.5 mm in diameter) by vortexing for 2 min (Minivortex, Sigma, Ireland). 0.8 mL of the homogenized mycelium suspension was used as inoculum in the fermentation experiment.

All the fermentation experiments were performed in 250 mL conical flasks containing 50 mL of the lipid production medium which was sterilized at 121ºC for 20 min. pH of the medium was adjusted to 6.0±0.5 using 1 N NaOH before sterilization and then confirmed after sterilization using a pH probe (Hanna instruments, Ireland). Flasks were incubated at 30±1ºC in the shaker incubator at 180 rpm under aerobic conditions. All the trials were conducted in triplicates. Regardless of the carbon sources used, pH values of the medium did not change significantly (5.8 - 6.5) during the whole fermentation period. However, when the same fungi *M. rouxii* was cultivated on potato processing wastewater, pH varied and rose from 6 at the beginning to 8 at end of the fermentation. This clearly shows that different culturing media would significantly affect the physiological responses of fungi.

Analytical methods

Flasks were removed from the shaker incubator at designed time intervals and subjected to analysis. The detailed procedures for biomass analysis and lipid extraction are given in Section 3.2.4.4 and 3.2.4.5, respectively. Profiles of long-chain fatty acids in microbial lipids were analyzed after direct transesterfication and then FAMEs were analysed using gas chromatography; Residual glucose was measured by the DNS method (Miller, 1959). Starch was measured by the phenol sulphuric acid method. Cellulose was measured according to the method adopted by Updegraff (1969). Briefly, 2 mL of properly diluted supernatant The procedure for obtaining supernatant should contain approximately 100 µg of cellulose, was added in a 15 mL glass tube, 10 mL of 67%

sulphuric acid was added and the tube was left undisturbed for 1 hr. After 1 hr, 1 mL solution was taken from the glass tube, added to a 250 mL conical flask, and diluted to 100 mL with distilled water. One mL of this diluted solution was transferred to a new 15 mL tube and 10 mL of anthrone reagent was added. After stirred, the tube was then heated in a water bath (90 ^{o}C) for 10 min. After cool down at the ambient temperature, the color intensity at 630 nm was measured using a spectrometer (Hach Lange, Ireland). Distilled water added with anthrone reagent was used as the blank for the spectrometry measurement with the procedure mentioned above. Cellulose with known mass (40 - 200 µg) was used to obtain the calibration curve for quantification of cellulose in the samples. α – amylase activity was measured using the method adopted by Bernfeld (1955). Cellulase activity was measured using the protocol described by Denison and Koehn (1977): briefly, Whatman No.1 filter paper was cut (7 mm diameter) using a paper punch and added into a 15 mL glass tube. Then, 0.5 mL of properly diluted sample supernatant was added to the tube. The mixture was placed in a water bath at 50 ^{o}C for 1 hr. Immediately after removing the mixture from the water bath, 0.5 mL of DNS reagent was added and the tube was heated again in a water bath at 90^{o}C for 5 min to terminate the enzyme activity. While the tube was warm 1 mL of Rochelle salt solution was added.

After cooling to ambient temperature, the aqueous volume in the tube was made up to 5 mL by adding distilled water. The absorbance of the mixture was measured at 540 nm using the spectrometer. The calibration curve was prepared with pure glucose with mass in the range of 50 µg - 1000 µg. One unit of enzyme activity (IU/mL) was expressed as mg of glucose released per min per mg of cellulose. Released glucose was measured by the DNS method.

Results and Discussion

Biomass growth, carbon source consumption and lipid production

The results of biomass growth and lipid production of *M. rouxii* on different carbon sources show that a noticeable biomass yield (X) was obtained (Table 1). Glucose supported biomass growth of *M. rouxii*, and almost complete consumption of glucose was observed leaving only 0.23 g/L of glucose in the medium within 5 days of cultivation (Fig. 1). It is obvious that most of oleaginous fungi can utilize glucose more rapidly than starch and cellulose. Starch seemed to be the best for supporting biomass production among the carbon sources tested, producing a higher biomass yield than glucose (15.5 g/L against 13.2 g/L).

Other researchers have found that starch is less efficient for biomass production for *Mucor* sp. (Ahmed *et al.*, 2006; Hansson and Dostálek, 1988), which is opposite to our research results. Papanikolaou *et al.*, (2007) observed similar results of increased biomass growth for cultures *C. echinulata* and *M. alpina* grown on starch over glucose. Uptake of starch was found to be significant when the initial starch concentration (Ci) was 30 g/L, and most of the soluble starch was utilized within 7 days of fermentation (Fig. 1), leaving only a small amount of starch in the medium (Table 1). Although more glucose was consumed than starch was, the biomass yield with respect to the consumption of the carbon source (YX/C) for glucose was less than for starch (Table 1). Since most of the starch was consumed when Ci was 30 g/L, in order to study the effect of increased starch concentrations on lipid accumulation (without altering the nitrogen concentration), Ci of 60 g/L was examined. In this case, almost 30% of starch was not consumed when the fermentation experiment was ended. On the

other hand, biomass production was not affected by the increase in the initial starch concentration; very slight reduction in biomass was observed when starch was increased from 30 g/L to 60 g/L (14.4 against 15.5 g/L). When the initial starch concentration was increased to 60 g/L, YX/C values were also lower than those when the carbon substrates were glucose or 30 g/L starch (Table 1).

Cellulose supported the biomass growth of *M.rouxii*. The biomass yield was up to 7.4 g/L at a cellulose concentration of 30 g/L. Two thirds of cellulose was not consumed even though the fermentation time was extended to 350 hr. YX/C values were much higher for cellulose than glucose and starch (Table 1).

This is the first report in biomass growth of *Mucor rouxii* on cellulose, since lignocellulosic raw materials are abundant and cheap in the nature. Lipid accumulation commenced after complete exhaustion of ammonium ions in the medium. Regardless of the carbon sources used complete exhaustion of ammonium nitrogen was observed at 68±5hr and the depletion pattern was similar for all carbon sources (Fig. 2).

The maximum lipid yield when glucose was the carbon substrate, 4.9 g/L, was higher than when starch was the carbon substrate, 3.9 g/L. The lipid contents in the dry biomass, YL/X, were 39.8% and 27.1% when the carbon substrates were 30 g/L glucose and starch, respectively.

Glucose, being a simple sugar, has supported the maximum lipid yield for most oleaginous microbes (Gema *et al.,* 2002). However, higher Lmax values for starch than for glucose were also observed when *C. echinulata* CCRS 3180 and *C. echinulata* ATHUM 4411 were grown on starch (Papanikolaou *et al.,* 2007).

Papanikolaou *et al.,* (2007) observed *C. echinulata* had higher YL/X values (28%) than *M. rouxii* (27.1%). When the concentration of starch was 60 g/L the maximum lipid yield, Lmax, was increased to 5.8 g/L (Table 1).

The research results show that cellulose did not support microbial lipid production. Although the biomass yield was up to 7.4 g/L, the lipid yield and the lipid content in biomass were much lower than for glucose and starch (Table 1).

The lipid yields with respect to carbon substrate consumption, YL/C, were almost similar for glucose and starch (both concentrations) as the carbon substrates (Table 1), but the value was very low for cellulose (0.01 g lipids/g cellulose consumed) than for other sources.

Although the fungi produced a considerable amount of cellulase, it seemed that the reducing sugar produced was used for biomass production. Another reason could be the errors in biomass measurement caused by the unconsumed cellulose.

The reason for the poor lipid yield on cellulose could be feedback inhibition by the substrate. Further studies should be conducted to optimize the cellulose concentration for obtaining high lipid yields.

In the experiment with 30 g/L glucose as the carbon source because a low amount of glucose was left in the medium, lipid turnover occurred. When the carbon source was 30 g/L starch, although starch was not utilized completely lipid turnover was observed. When 30 g/L cellulose and 60 g/L starch were used as the carbon substrates, lipid turnover did not take place probably due to the presence of excess organic carbon substrates in the medium (Fig. 3).

Fig.1 Utilization of different carbon sources by *M. rouxii*

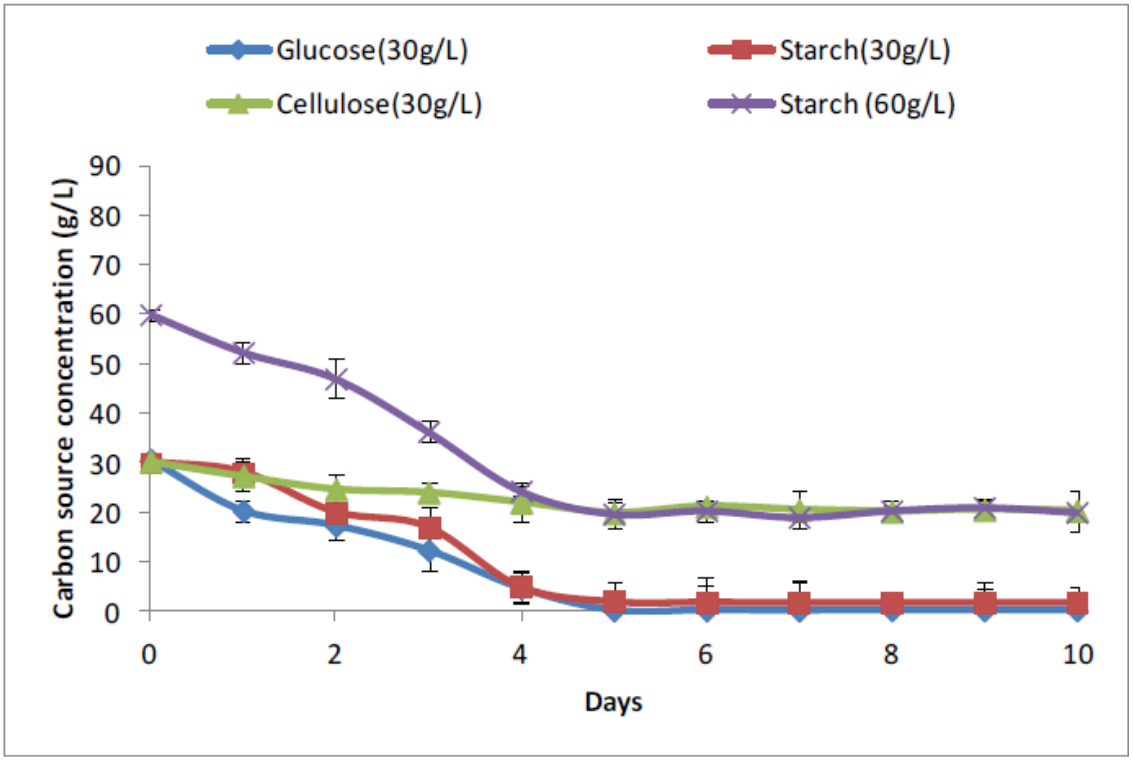

Fig.2 Depletion of ammonium nitrogen concentrations in different carbon sources

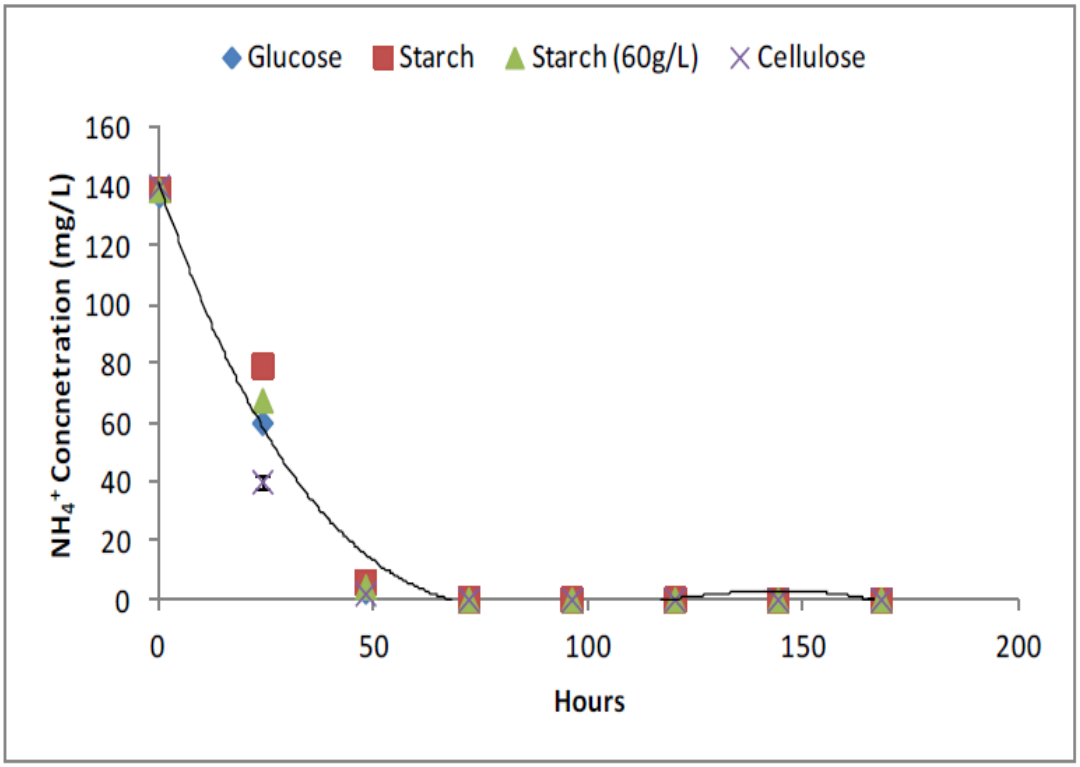

Fig.3 Lipid Production by *M. rouxii* with different carbon sources

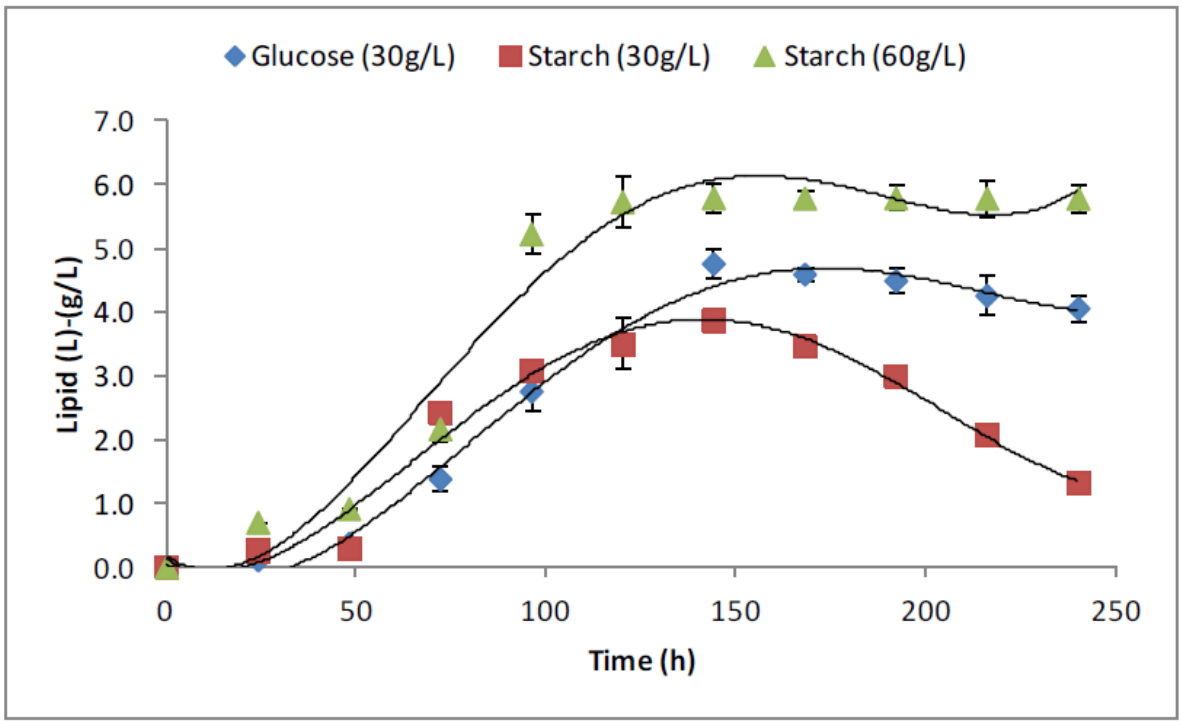

Fig.4 Secretion of amylase and cellulases under different carbon sources

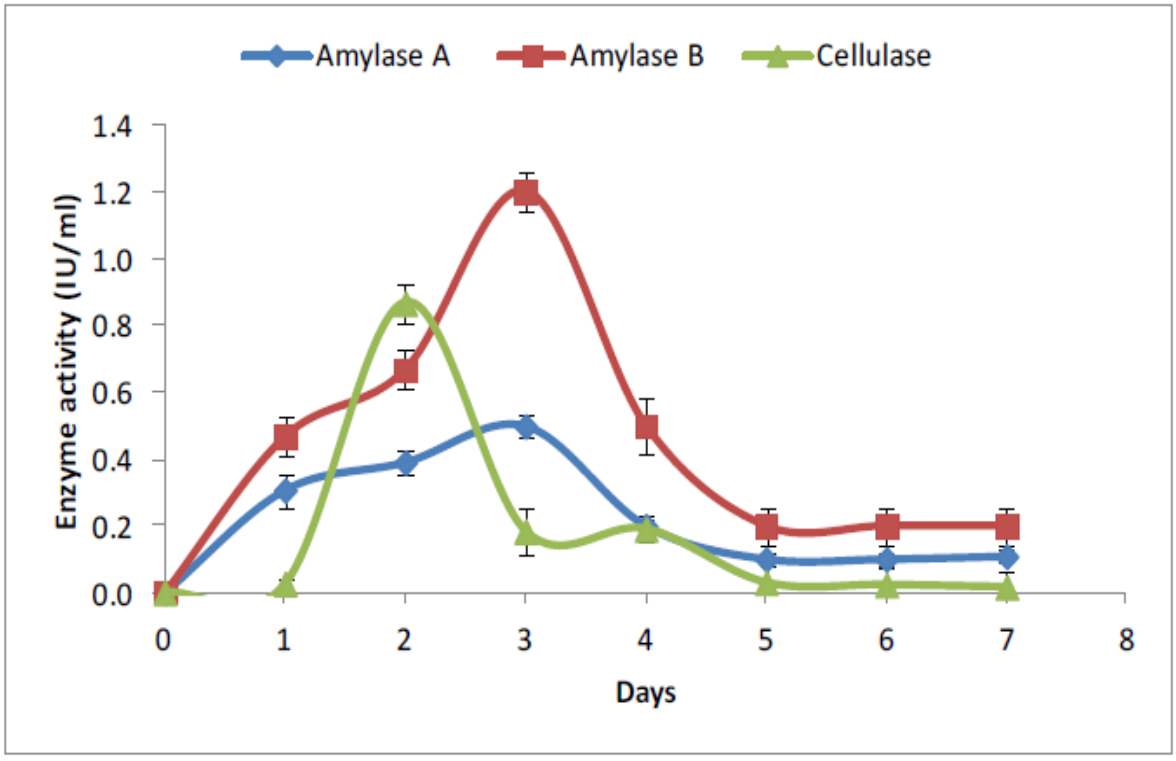

Table.1 Growth and lipid production of *M. rouxii* on different carbon sources

Carbon source	Ci (g/L)	Time (hr)	X (g/L)	Lmax (g/L)	Cf (g/L)	Y X/C (g/L)	Lipid content (%,wt/wt)	YL/C (g/g)
Glucose	30	144	12.3±0.91	4.9±0.4	0.23±0.01	0.44	39.8	0.16
Starch	30	144	14.4.±1.2	3.9±0.2	1.8±0.2	0.51	27.1	0.14
	60	144	13.4±1.1	5.8±0.5	20±0.98	0.34	43.3	0.15
Cellulose	30	350	7.4±0.3	0.05±0.001	20.5±1.2	0.78	0.7	0.01

Calculation of parameters such as YX/C, lipid content, YL/C are given in Section 3.2.4.6, Chapter 3
Ci: initial substrate concentration; Cf: substrate concentration at time t.

Table.2 Specific substrate uptake rates of *M. rouxii* Su (g substrate/g microorganism.h)

Carbon source	Fermentation time	Average substrate uptake rate S*u* (g/g.h)
Glucose (30 g/L)	24	0.341
	48	0.109
	72	0.065
Starch (30 g/L)	24	0.076
	48	0.024
	72	0.048
Starch (60 g/L)	24	0.063
	48	0.082
	72	0.046
Cellulose (30 g/L)	24	0.053
	48	0.006
	72	0.01

Substrate uptake rate and hydrolytic activity of *M.rouxii*

Specific uptake rates for different carbon sources by *M. rouxii* were calculated which TSS was replaced with respective carbon substrate, and the results are presented in Table 2. At the initial carbon substrate concentration of 30 g/L, the specific substrate uptake rate of glucose was higher than those of starch and cellulose. In the first 24 hr, the specific substrate uptake rate for glucose was much higher and the rate was reduced after 48 hrs (Table 2) and the complete exhaustion of glucose was found within 5 days of fermentation (Fig. 1). This rapid uptake rate within 24 h indicates that glucose was channeled into cells for lipid synthesis. Specific uptake rates for starch at both levels and for cellulose were lower than that of glucose in the first 24 hrs. This suggests that complex carbon sources cannot be uptaken directly as glucose by *M. rouxii*. It is reported that the uptake of complex carbon sources by oleaginous mucorales is greatly influenced by the secretion of hydrolytic enzymes (Papanikolaou *et al.*, 2010; Papanikolaou *et al.*, 2007).

Enzyme secretion was observed in the experiment when cultivated on starch and cellulose (Fig. 4). When the carbon substrate was 30 g/L starch, amylase secretion started from the 1st day of fermentation with 0.3 IU/mL, the maximum amylase activity was 0.5 IU/mL on the 5th day and thereafter declined. A similar pattern of amylase secretion was observed with the maximum

activity of 1.2 IU/mL when the starch concentration was 60 g/L (Fig. 4). The obtained amylase values (0.5 and 1.2 IU/mL) in this study were high, in comparison with that of the fungal culture *M. isabelliana ATHUM 2935*: when it was grown on starch, the maximum amylase secreted was 0.12 IU/mL within 24 hr of fermentation (Papanikolaou *et al.,* 2007). This suggests that *M. rouxii*is better than *M. isabelliana ATHUM 2935* for starch hydrolysis. In contrast, the production of cellulase was different from amylase. The quantity of cellulase secretion was very low during the first day, increased from the 2nd day and reached the maximum activity on the 4th day (0.19 IU/mL).

The concentrations of reducing sugars in the medium containing 30 g/L starch were negligible (the maximum concentration was 0.12 g/L of glucose equivalents) after the 6th day, suggesting that most of the reducing sugars released were being utilized simultaneously. It is also worth mentioning the fact that there was no secretion of amylase or cellulase measured when glucose was the carbon source. Hence it is revealed that enzyme secretion of *M. rouxii* is substrate specific.

Composition of microbial lipids produced under different carbon sources

The composition of long-chain fatty acids in microbial lipids is given in Table 3. With the three carbon sources tested, oleic acid (C18:1) was the predominant long-chain fatty acid present in microbial lipids. This phenomenon was also observed by other researchers (Ahmed *et al.,* 2006; Papanikolaou *et al.,* 2010; Somashekar *et al.,* 2003): for oleaginous *Zygomycetes* cultured with renewable carbon sources oleic acid is the predominant long-chain fatty acid contained in microbial lipids. This may be due to an icr s d Δ9 dehydrogenase activity in the oleaginous microorganisms. Then palmitic acid (C16:0) shared second most part of the lipid produced by *M. rouxii*, followed by stearic acid (C18:0), linolenic acid and GLA. Higher contents of GLA were found in the microbial lipids with starch and cellulose as the carbon substrates than with glucose as the carbon source. However for the given glucose concentration contents of GLA were higher than other mucaraceous cultures. Starch had higher GLA than glucose and the concentration was 11.2 % when the concentration of starch was 60 g/L. Similar results of GLA content was found in *Mucor* sp. RRL001 when they were cultivated with tapioca starch (Ahmed *et al.,* 2006). The comparison of GLA contents in microbial lipids of *Mucor rouxii* and *A. oryzae* which is non mucaraceous shows that much higher percentages of GLA was produced by mucaraceous fungi (*Mucor rouxii*) (Table 3) than non mucaraceous species, suggesting that mucaraceous fungi are best GLA producers.

In this chapter biochemical behaviors of *M. rouxii* were studied with three carbon substrates – glucose, starch and cellulose. All the carbon sources supported biomass growth; particularly, starch had a higher biomass growth than others. Interestingly cellulose supported good biomass growth suggesting further research should be conducted on this carbon source. Glucose had a higher lipids yield than starch did; cellulose did not support lipid production for *M. rouxii.* A high starch concentration supported a high GLA content in lipids; the maximum GLA content of 11.2% was found with 60 g/L of starch as the carbon substrate

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