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ORIGINAL PAPER

Sugarcane molasses and yeast powder used in the Fructooligosaccharides production by *Aspergillus japonicus*-FCL 119T and *Aspergillus niger* ATCC 20611

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Abstract Different concentrations of sucrose (3–25% w/v) and peptone (2–5% w/v) were studied in the formulation of media during the cultivation of *Aspergillus japonicus*-FCL 119T and *Aspergillus niger* ATCC 20611. Moreover, cane molasses (3.5–17.5% w/v total sugar) and yeast powder (1.5–5% w/v) were used as alternative nutrients for both strains' cultivation. These media were formulated for analysis of cellular growth, β-Fructosyltransferase and Fructooligosaccharides (FOS) production. Transfructosylating activity (U_t) and FOS production were analyzed by HPLC. The highest enzyme production by both the strains was 3% (w/v) sucrose and 3% (w/v) peptone, or 3.5% (w/v) total sugars present in cane molasses and 1.5% (w/v) yeast powder. Cane molasses and yeast powder were as good as sucrose and peptone in the enzyme and FOS (around 60% w/w) production by studied strains.

Keywords Fructooligosaccharides · *Aspergillus japonicus* · *Aspergillus niger* · Cane molasses · Yeast powder

Introduction

Fructooligosaccharides (FOS) are important for their functional properties and sweetening power. FOS are low calorie and non-cariogenic sweeteners, provide relief from constipation, decrease total cholesterol and lipid in serum, promote animal growth and consecutively improve the intestinal microflora, act as a growth factor for *Bifidobacterium* and *Lactobacillus*. [3, 7, 15, 23]. Moreover, they replace the sucrose in the flavor, viscosity, humidity, body, browning and freezing points of alimentary products [10, 13, 19].

FOS are derived from sucrose using microbial or many plant enzymes and found in trace amounts as natural components in fruits, vegetables and honey [23]. They are composed of sucrose attached by a β (2-1) linkage to one to three fructose units and are called, 1-kestose, nystose and fructosyl nystose, respectively [19].

β-Fructofuranosidase (β-FFase, EC 3.2.1.26) enzyme with β-Fructosyltransferase (β-FTase, EC 2.4.1.9) activity derived from *Aspergillus niger* ATCC 20611 was considered more appropriate for industrial production, being able to convert sucrose into FOS with 60% of yield [13]. However, some bacterial levansucrases (EC 2.4.1.10) that originated from *Gluconocetobacter diazotrophicus*, *Acetobacter diazotrophicus* and *Zymomonas mobilis* have been reported to produce FOS such as 1-Kestose and nystose [1, 2, 20, 21].

Sucrose and yeast extract or peptone were commonly used by researchers for production of β-FTase and biomass [4, 9, 10, 16]. According to Chen [3], sucrose and yeast extract were the key nutritional factors affecting enzyme production; improved media containing 24% sucrose and 2.75% yeast extract increased enzyme production by 180% by *Aspergillus japonicus* TIT-90076. Hayashi et al. [9] after various nitrogen sources used for enzyme production by *Aspergillus japonicus* MU-2, showed that yeast extract was the best but peptone was also good. These researches showed that among carbon sources used for enzyme production by MU-2 strain, sucrose was the best, and good

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enzyme production was also achieved using glucose and soluble starch.

For industrial production of FOS, improvement in biomass and enzyme production by alternative nutritional medium is very important to reduce the cost of carbon and nitrogen sources. Cane molasses and yeast powder are abundant and inexpensive; they are residues of sugar and alcohol industry. Their use as nutrients for FOS technology was investigated in this work and compared with traditional nutrients. Strains of *Aspergillus japonicus* FCL 119T isolated by Cruz et al. [4] and *Aspergillus niger* ATCC 20611 (control strain) selected by Hidaka et al. [10], both with great potential for industrial FOS production, were used in the cultivation with different media for production of β-FTase.

Materials and methods

Microorganisms and culture medium

Aspergillus japonicus-FCL 119T and *Aspergillus niger* ATCC 20611 (control strain) were maintained on Potato Dextrose Agar slants (PDA-Difco) at 4°C, pH 5.5 in the Biochemistry and Microbiology laboratory of São Paulo State University/UNESP/Assis-Brazil. The inoculum was prepared by transferring 0.5 ml spore suspension (1.8×10^7 spore) in 125 ml Erlenmeyer flasks containing 50 ml of medium composed of different concentrations of commercial sucrose (3, 5, 10, 15, 20 and 25% w/v) as carbon source. The media were also formulated using B cane molasses with 68.6% (w/v) total sugar (61.7% sucrose, 4.3% glucose and 2.6% fructose—from USINA NOVA AMÉRICA-UNA/Tarumã—Brazil) as alternative carbon sources. In these media, the molasses was diluted with distilled water to 3.5, 7, 10.5, 14 and 17.5% w/v of total sugars. Peptone-Difco (2, 3, 4, and 5% w/v) or yeast powder-UNA (1.5, 2, 3, 4 and 5% w/v) were utilized as nitrogen sources. In all media were fixed the salts concentration 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.5% K_2HPO_4, 0.2% $NaNO_3$, pH 5.0. The flasks were agitated in an orbital shaker (Tecnal TE421/SP/Brazil), at 160 rev min $^{-1}$ for 60 h at 28°C. Table 1 shows the 17 media tested. At the end of 60 h of culture fermentation, the biomass was harvested by filtration with filter paper (Whatman). Dry cell weight was determined by drying the pellets to constant weight (for 4 days) at 105 °C in a hot air oven (Tecnal/SP/Brazil).

Table 1 Media tested during the culture of *Aspergillus japonicus* FCL 119T with *Aspergillus niger* ATCC 20611 as control strain

Medium	Nitrogen source		Carbon source	
	Peptone % (w/v)	Yeast powder[a] % (w/v)	Sucrose % (w/v)	Sugars in cane molasses[b] % (w/v)
1	2	0	3	0
2	3	0	3	0
3	4	0	3	0
4	5	0	3	0
5	3	0	5	0
6	3	0	10	0
7	3	0	15	0
8	3	0	20	0
9	3	0	25	0
10	0	1.5	0	3.5
11	0	3.0	0	3.5
12	0	4.5	0	3.5
13	0	1.5	0	3.5
14	0	1.5	0	7.0
15	0	1.5	0	10.5
16	0	1.5	0	14.0
17	0	1.5	0	17.5

Fermentation time was 60 h at 28°C at 160 rev min $^{-1}$

[a] Yeast powder composition**:** protein 40%, mineral says (K, Na, Ca, P,Mg, S, SO_4, Fe, Co) 5.44%, lipids 0.8%, fibers 1.08% e vitamins at 10 g: B1(0.264 mg), B2(0.592 mg), Niacin (5.5 mg), pentatonic acid (0.018 mg), pyridoxine (0.041 mg), folic acid (0.128 mg), Biotin (0.224 mg), p-amid benzoic acid (0.358 mg), coline (37 mg) and inosytol 36 mg.(UNA)

[b] Molasses composition: water (11%), sucrose (61.7%), glucose (4.3%), fructose (2.6%), nitrogen materials, free and agree acids, and gum (8%), CaO (2.3%), MgO (0.88%), P_2O_5 (0.15%), SO_2 (2.1%), clorets (0.55%), SiO_2 (0.44%), Al_2O_3 (0.88%)Fe_2O_3 (0.88%), K_2O (3%), Na_2O (0.8%)

FOS production

FOS production was determined as described by Cruz et al. [4], with minor modifications. The reaction mixture contained 0.3 g of intracellular crude enzyme and 4.5 ml of 40% (w/v) sucrose solution in McIlvaine buffer (150 mM, pH 5.5), and incubated in water bath (Tecnal/SP/Brazil) at 50°C. After 90 min, the reaction was stopped by treatment in boiling water for 5 min and the proteins were precipitated by addition 0.2 ml barium hydroxide (5.2% w/v) and 0.2 ml zinc sulphate (5% w/v), followed by centrifugation 1,600×*g* for 10 min. FOS yield (% w/w) was calculated from FOS produced (g)/initial concentration of sucrose (g) × 100.

Analytical procedure

Quantitative analysis of the products of enzyme reation was done using HPLC (Agilent Technology, Mod. HP 1100) with an isocratic pump (Mod. G1310) equipped with column (shodex-NH_2, 250 nm × 4.6 mm) operated at 35°C and with a refractive index detector (RID-6A). A system composed of acetonitrile (Merck) and Milli-Q (Millipore/Gradient JBRQ)

water (75/25) as solvent and a flow rate of 2 ml min^{-1}. The samples were diluted appropriately and filtered through a membrane filter with a pore size of 0.45 μm (Millipore) before injection. Glucose, fructose, sucrose (P.A. Labsynth/Campinas/Brazil), 1-kestose, nystose and fructosyl-nystose (Waco pure chemical Ltd.), all with known concentration, were used as standards of the samples of FOS. The FOS were quantified through the method of external standardization.

β-Fructosyltransferase activity

Transfructosylating activity (U_t) was determined from the amount of fructose transferred for sucrose [4]. One unit of this activity was defined as the amount of enzyme required to transfer 1 μmol of fructose per minute in the assay conditions.

Reproducibility of results

The experiments for the cellular growth were performed five times, β-Fructofuranosidase activity three times and shown as averages and standard deviations. Statistical analysis (Graphad Instat, Rutgers University, Camden, New Jersey) was carried out comparing the averages of the cellular growth (ANOVA and Tukey tests with $p < 0.05$ considered significantly different).

Results and discussion

Cellular growth, β-FTase and FOS production on sucrose and peptone medium

The effect of sucrose concentration on cellular growth was investigated as shown in Fig. 1. The different concentrations of sucrose had significant influence (Anova, $p < 0.05$) on the fungus strains growth. *Aspergillus niger* ATCC 20611 strain showed cellular growth until the 20% (w/v) sucrose, after this concentration the growth stopped. The increase in fungal growth was 99% from 5 to 20% (w/v) of this sugar. When 5% (w/v) sucrose was replaced for 15% (w/v) occured a significant (Tukey, $p < 0.05$) increase of 41.5% in dry biomass of the *Aspergillus japonicus* FCL 119T. The control strain was 57% higher in biomass formation than FCL119T strain at 20% (w/v) sucrose. Jung et al. [12] studied the ideal concentration of sucrose for the cellular growth for *Aureobasidium pullulans* and verified that the best concentration for the biomass production was 10% (w/v). However, in this paper it is reported that 15 and 20% (w/v) sucrose are the best concentrations

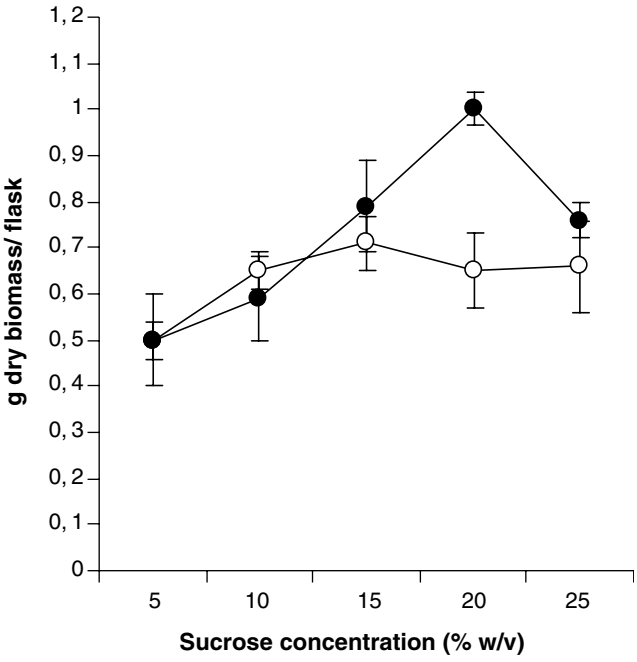

Fig. 1 Effect of sucrose concentration on dry biomass production by *open circle A. japonicus*-FCL 119T and *filled circle A. niger* ATCC 20611. As nitrogen source 3% (w/v) peptone

for cellular growth by FCL 119T and ATCC 20611 strains, respectively.

The increase of the sucrose concentration caused a linear decrease in the β-FTase activity after cultivation of the two strains (Fig. 2). The optimum concentration of sucrose for β-FTase production was 5% (w/v) by the two strains. Jung et al. [12] evidenced that the best concentration of sucrose for the enzymatic production by *Aureobasidium pullulans* occurred above 25% (w/v). Hayashi et al. [9] had concluded that a good concentration of sucrose for the production of β-FTase by *Aspergillus japonicus* (Mu-2) was 20% (w/v). According to Chen [3], the ideal concentration of sucrose for enzymatic production by *A. japonicus* Tit-90076 is 24% (w/v). However, Hidaka et al. [10] and Cruz et al. [4] showed high production of the fungal enzyme at 5.0 and 2.5 % (w/v) sucrose, respectively, both reaching 60% FOS yield (w/w). The use of low concentration of sucrose resulting in high production of β-FTase is important because it decreases the cost of the process.

With 5% (w/v) sucrose, the concentration FOS yield (% w/w) reached 57 and 53% by ATCC 20611 and FCL 119T strains, respectively. According to literature the maximum FOS yield is 60% (w/w) [4, 13, 16], close yields were obtained in the present work. Sangeetha et al. [16] working in similar concentration of sucrose (6.4% w/v) obtained the best FOS yield (58% w/w) by *Aspergillus oryzae* CFR 2002.

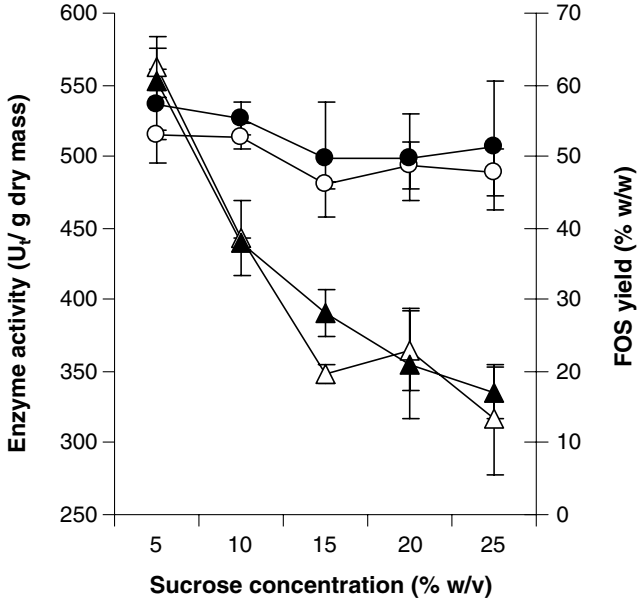

Fig. 2 Effect of sucrose concentration on U_t and FOS yield. *open triangle* U_t and *open circle* FOS yield of the *A. japonicus*-FCL 119T. *filled triangle* U_t and *filled circle* FOS yield of the *A. niger* ATCC 20611. As nitrogen source 3% (w/v) peptone

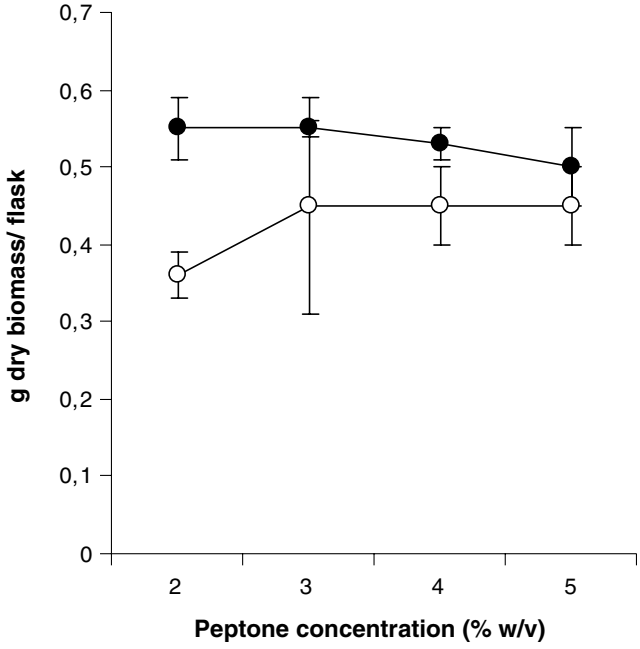

Fig. 3 Effect of peptone concentration on dry biomass production by *open circle A. japonicus*-FCL 119T and *filled circle A. niger* ATCC 20611. As carbon source 3% (w/v) sucrose

This work shows that lower concentration of carbon source produces higher enzyme and lower cellular growth. Smith et al. [18] and Jung et al. [12] showed that an important factor in the β-FTase production was not final cell mass but sucrose concentration. These factors suggested that the highest cellular growth of the fungus strains deviated more carbon and nitrogen for cellular mass decreasing enzyme production.

The change in the peptone concentration had no significant influence on the production of biomass by two strains (Fig. 3). The ATCC 20611 strain showed higher cellular growth than FCL 119T only at 2% (w/v) peptone; this significant (Tukey, $p < 0.05$) difference was 36%.

Fig. 4 shows the influence of peptone in the β-FTase and FOS production. The maximum enzyme production was in 3% peptone with FOS yield around 60% (w/w) by both strains. The literature showed 1.5–3.0% (w/v) yeast extract as the best concentrations of the nitrogen source for β-FTase production by different strains of *Aspergillus japonicus* [3, 9]. Fig. 4 shows that 3% (w/v) sucrose produced higher FOS than 5%(w/v) (Fig. 2) at 3% (w/v) peptone.

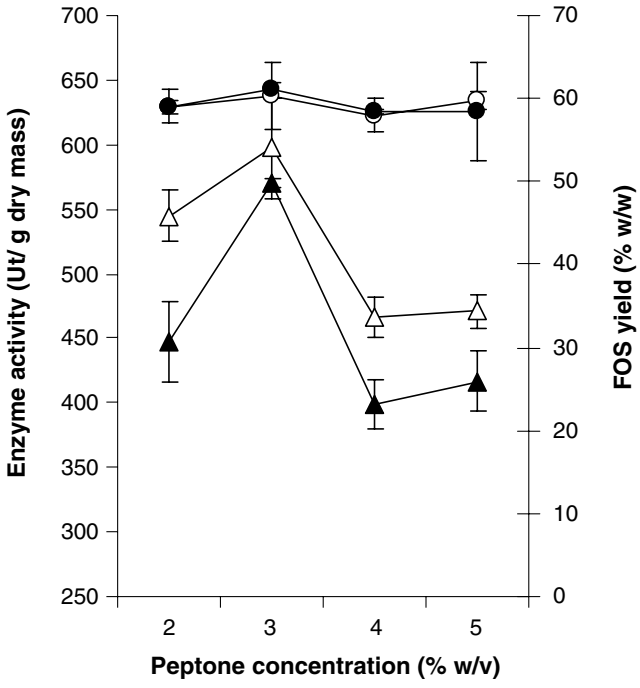

Fig. 4 Effect of peptone concentration on U_t and FOS yield. *open triangle* U_t and *open circle* FOS yield of the *A. japonicus*-FCL 119T. *filled triangle* U_t and *filled circle* FOS yield of the *A. niger* ATCC 20611. As carbon source 3.0% (w/v) sucrose

Cellular growth, β-FTase and FOS production on alternative medium

Figure 5 is shows the effect of the concentration of total sugars present in cane molasses on cellular growth of the ATCC 20611 and FCL 119T strains. The sugar concentration significantly influenced (ANOVA, $p = 0.0001$) the cellular growth of the studied strains.

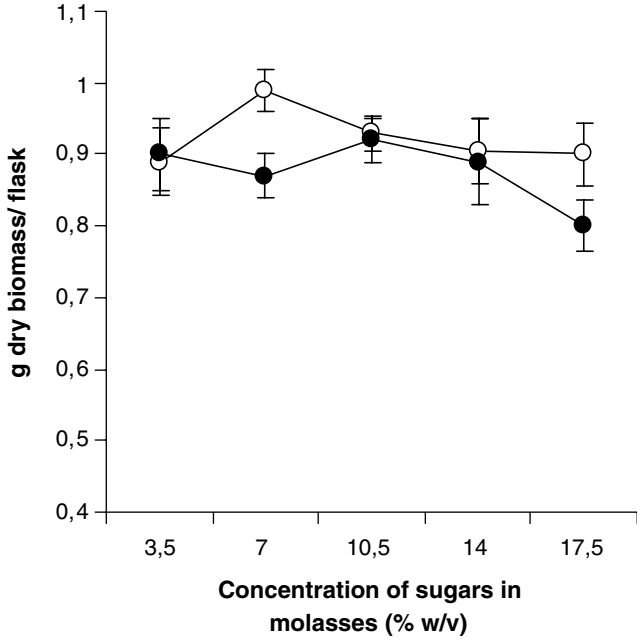

Fig. 5 Effect of the concentration of total sugars in cane molasses on dry biomass production by *open circle A. japonicus*-FCL 119T and *filled circle A. niger* ATCC 20611. As nitrogen source 1.5% (w/v) yeast powder

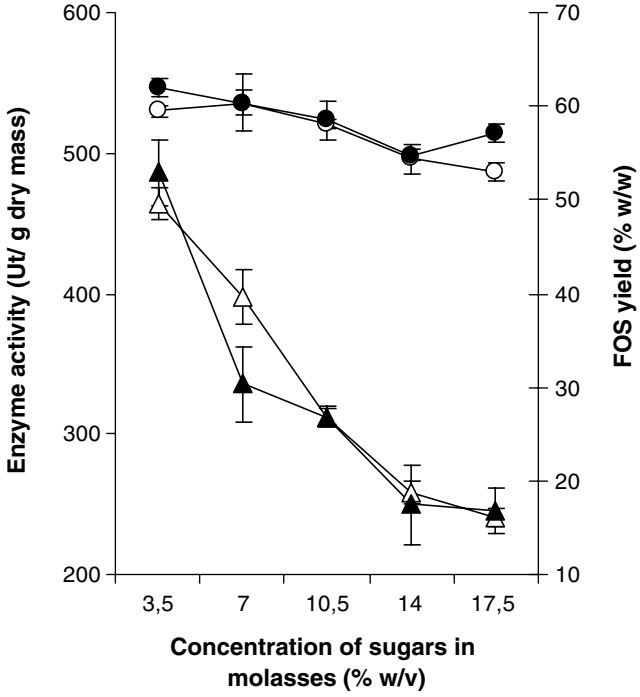

Fig. 6 Effect of the concentration of total sugars in cane molasses on U_t and FOS yield. *open triangle* U_t and *open circle* FOS yield of the *A. japonicus* FCL 119T. *filled triangle* U_t and *filled circle* FOS yield of the *A. niger* ATCC 20611. As nitrogen source 1.5% (w/v) yeast powder

The fungus growth occurred in 3.5–10.5% (w/v) sugars, and higher up 10.5% (w/v) was verified the reduction of biomass. The FCL 119T strain obtained 14.5% more cellular growth than control at 7% (w/v) sugars. The optimum concentrations for cellular growth of the FCL 119T and control strains were 7.0 and 10.5 % (w/v), respectively.

The optimum concentration of sugars in cane molasses for cellular growth of the two strains was in the average 8.75% (w/v) and for commercial sucrose 17.5% (w/v). This difference in the concentration of sugars in molasses and commercial sucrose occurred probably because of the impurities on alternative carbon source as SO_2 (1,071–5,400 mg/l), clorets (280–1,400 mg/l) and Al_2O_3 (449–2,251 mg/l). The SO_2 is used as disinfectant specially in wine production [22]. According to Hinze and Holzer [11], the energetic metabolism of yeast was inhibited with 400.5 mg sulfite/l decreasing the ATP level in the cells. Schimz [17] mentioned that high sulfite concentration decreased cell viability and yeast death. The clorets are potent antimicrobial agents and are utilized in municipal water treatment. Clorets act as HOCl when addeded in water, this acid is a potent agent of oxidation and it inhibits the activity of various cellular enzymes. [22]. The Al^{3+} ion is toxic for microrganisms, including bacteria, fungus and green seaweed [5, 6, 8, 24].

The effect of the concentration of sugars in cane molasses on enzyme and FOS production by two studied strains was investigated. The optimum sugar concentration for β-FTase activity and FOS yield was 3.5% (w/v) by both strains (Fig. 6). In this concentration, FOS yield was around 60% (w/w) by two strains. Above 3.5% (w/v) had strong decrease in enzyme activity, it reached 49% at 17.5% (w/v) sugars, probably it also occurred because this concentration increased the presence of inhibitory salts.

The influence of yeast powder concentration on cellular growth by FCL 119T and control strains was observed. The highest yeast powder concentrations increased significantly (Anova, $p < 0.05$) the biomass of the two strains studied (Fig. 7). The cellular growth of FCL 119T and control strains reached 67 and 100%, respectively, in 4.5% (w/v) yeast powder when compared with 1.5% (w/v) this nutrient. ATCC 20611 strain growth was 40% higher than the other strain in 3 and 4.5% (w/v) this nitrogen source.

Yeast powder produced higher cellular growth than peptone (Figs. 3, 7) for two strains. This increase in biomass could be due to the vitamins and minerals present in yeast powder (Table 1). *Aspergillus niger* is not dependent of rich medium [14] but theses components could be used as stimulant nutrients of growth.

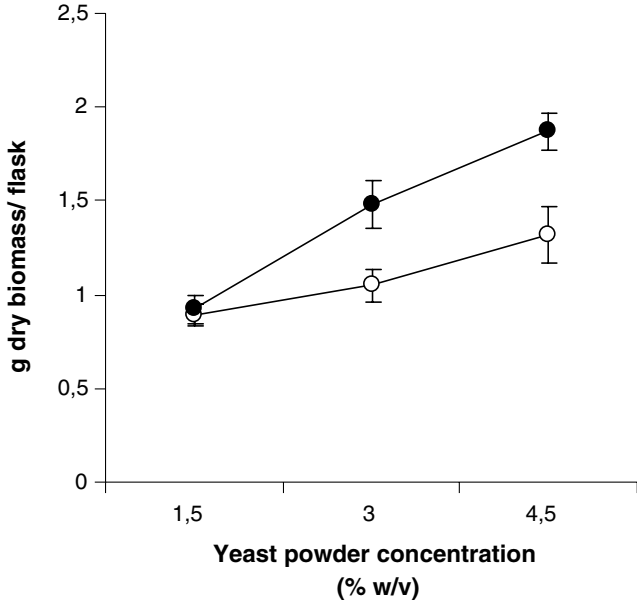

Fig. 7 Effect of yeast powder concentration on dry biomass production by *open circle A. japonicus* FCL 119T and *filled circle A. niger* ATCC 20611. As carbon source 3.5% (w/v) sugars in molasses

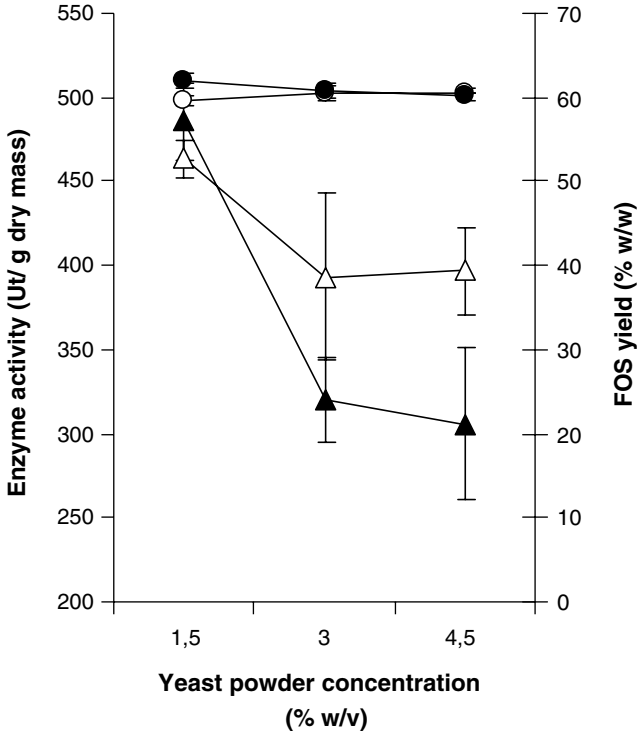

Fig. 8 Effect of yeast powder concentration on U_t and FOS yield. *open triangle* U_t and *open circle* FOS yield of the *A. japonicus*-FCL 119T.*filled triangle* U_t and *filled circle* FOS yield of the *A. niger* ATCC 20611. As carbon source 3.5% (w/v) sugars in molasses

The optimum concentration of yeast powder for β-FTase production was 1.5% (w/v) by strains studied, but the FOS yield were about 60% (w/w) for 1.5 to 4.5% (w/v) concentrations (Fig. 8). It could have occurred because the highest cellular growth probably compensated for the lowest enzyme cell production.

The best culture media obtained in the work for β-FTase production were formulated with 3% (w/v) sucrose and 3% (w/v) peptone, or 3.5% (w/v) total sugars present in molasses and 1.5% (w/v) yeast powder, both with salts as complement. In these conditions, the FOS yield reached the maximum as showed in the literature.

Since cane molasses and yeast powder are residues from sugar and ethanol industries, they are cheaper than sucrose and peptone or yeast extract and could be used for industrial purposes in the FOS production. Moreover, this alternative medium was efficient for β-FTase production by two strains that have great potential for industrial FOS production.

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