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Tiger Nut (*Cyperus esculentus*) Milk Byproduct and Corn Steep Liquor for Biosurfactant production by *Yarrowia lipolytica*

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The use of agroindustrial wastes for biosurfactant production is advantageous since the raw material represents a great part of the costs to obtain this bio-product. Low cost raw materials (Tiger nut fibre – TNF and corn steep liquor – CSL) were evaluated as potential carbon and nitrogen sources for biosurfactant production by *Yarrowia lipolytica* IMUFRJ 50682. It was demonstrated that it is possible to produce biosurfactant using corn steep liquor or tiger nut fiber in medium containing ammonium sulfate, with emulsification indexes (EI) around 40% for each residue. The culture medium containing ammonium sulfate, corn steep liquor and tiger nut fiber presented the same EI (60%) as the medium containing glucose, yeast extract, glycerol and ammonium sulfate (Control medium). This is the first report of the use of tiger nut by-product for biosurfactant production. It was estimated that the cost for ammonium sulfate, corn steep liquor and tiger nut fiber medium is one third the cost of control medium.

1. Introduction

Biosurfactants are molecules with amphipathic character able to reduce interfacial and superficial tension, as chemical surfactants. They offer several advantages over their chemical counterparts, such as: biodegradability, low toxicity, ecological acceptability, which makes them a good alternative for application in several industries. Biosurfactants can be produced by microorganisms during cell growth or in the stationary phase of growth (Makkar et al., 2011).

Although biosurfactants exhibit such important advantages, they have not been yet employed extensively in industry because of relatively high production costs. An alternative to reduce the production costs is to use agro-industrial wastes as a source of carbon and/or nitrogen for microorganisms. Besides, the use of these residues is good for the reduction of environmental impacts (Fontes et al., 2012). Different renewable resource and agro industrial residuals have been reported to be used for biosurfactant production, such as olive oil mill effluent (Mercade et al., 1994), cashew apple juice, glycerine (Fontes et al., 2012), distillery residues and whey (Dubey et al., 2001).

Tiger nuts (*Cyperus esculentus*) produce tubers with a yellowish core, sweet to the palate, with excellent concentrations of proteins, vitamins, lipids, fibers, among others. It is mainly used to produce 'horchata de chufa' (tiger nut milk) (Sanchez-Zapata et al., 2009). This sweetened water extract of tiger nut tubers is a very popular drink in Spain and has a potential market in other countries. In their production, two residues are generated: the Tiger nuts fiber (TNF) and the liquid residue, tiger nuts liquid co-product (TNLC) (Sanches-Zapata et al., 2012). Until now, the most common disposal method of 'horchata' co-product (solid and liquid) had been its use as an organic mass for combustion, composting and animal feed (Sanchez-Zapata et al.,

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2009). Researchers have been evaluating its use as food ingredients (Sanchez-Zapata et al., 2012), but its valorization for biosurfactant production hasn't been studied yet.
Corn steep liquor (CSL), a major by-product of the corn wet-milling industry, is also an inexpensive source of nutrients available on a large scale, and it has been successfully used for a variety of fermentations (De Azeredo et al., 2006). It is frequently used as nutrient replacement to yeast extract (Maddipati et al., 2011).
Among yeasts, *Yarrowia lipolytica* have been studied and used for the production of biosurfactants with the advantage of having GRAS (generally regarded as safe) status offering no risks of toxicity and pathogenicity (Bath and Gaillard, 1997). Additionally, this specie has the ability to use several agro-industrial wastes (Fontes et al., 2012).
Therefore, this work aims at evaluating the use of TNF and CSL as alternative sources of carbon and nitrogen for the production of biosurfactant by *Y. lipolytica* IMUFRJ 50682, reducing production costs for the development of a biotechnological process.

2. Materials and Methods

2.1 Microorganism and Materials

A wild type strain of *Yarrowia lipolytica* (IMUFRJ 50682) was employed (Hagler and Hagler-Mendonça, 1981) and kept at 4º C on YPD-agar medium.
The following materials were used: Peptone and yeast extract (Oxoid-Hampshire, UK), glucose, agar-agar, ammonium sulfate and glycerol (Vetec-R.J., Brazil), hexadecane (Sigma-Aldrich CO, USA).
The corn steep liquor (CSL) was donated by Ingredion Brasil (composition in Table 1) and tiger nut fiber (TNF, Table 1) was obtained by the extraction of tiger nut milk produced by Costa Neto et al. (2017). Both residues were stored in sealed containers and kept in a refrigerator at 4 ° C.

Table 1: Corn steep liquor (Ingredion Brasil) and tiger nut fiber (Sanches-Zapata et al., 2012) compositions

Component	Amount (%)	Component	Amount (mg/kg)	Component	Amount (mg/kg)
Corn Steep Liquor					
Nitogen	3.41	Iron	647.5	Biotin	0.3
Phosphorus	1.12	Manganese	57.5	Colin	3,500
Potassium	2.9	Cupper	22.5	Inositol	6,000
Calcium	2	Zinc	152.5	Niacin	80
Magnesium	0.95	TOC	16.2 %	Pantothenic acid	15
Sulphur	0.25			Pyridoxine	9
Boron	0.08			Riboflavin	6
Sodium	0.08			Thymine	3
Tiger nut fiber					
Humidity	61.23				
Protein	1.75				
Lipid	8.85				
Ash	0.99				
Total dietary fibre	59.71				

TOC: Total organic carbon

2.2 Biosurfactant production

For inoculum conditions, cells were cultivated at 28º C in a rotary shaker at 160 rpm, in 500 ml shake flasks containing 200 ml of YPD medium (w/p: yeast extract, 1%, peptone, 0.64%; glucose, 2%). After 72 h of cultivation, these cells were used in sufficient amount to inoculate 1 mg of cells (dry weight) per ml of biosurfactant production media.
Biosurfactant production was carried out in 1000 ml shake flasks, containing 500 ml of the culture medium, in a rotary shaker at 28º C and 250 rpm for 96 h. Samples were collected at time-defined intervals and submitted to analysis.
The residues (TNF and CSL) were disinfected by exposure to ultraviolet radiation for 20 minutes. As reference, a production medium optimized by Fontes et al. (2012) for the production of biosurfactant by *Y. lipolytica* IMUFRJ 50682, herein named control (CT). The media containing the residues had the yeast extract and the carbon sources replaced by the residues, maintaining or not ammonium sulfate, as shown in Table 2.

Table 2: Media composition for biosurfactant production by Yarrowia lipolyica

Media	Components (g/L)					
	Glucose	Glycerol	Yeast Extract	Ammonium sulfate	TNF	CSL
CT	40	20	0.5	10	-	-
AmSft	0	0	0	10	-	-
TNF + AmSft	0	0	0	10	10	-
CSL + AmSft	0	0	0	10	-	2.5
TNF + CSL + AmSft	0	0	0	10	10	2.5

CT: control; AmSft: ammonium sulfate; TNF: tiger nut fiber; CSL: corn steep liquor

2.3 Analytical methods

Cell concentration was followed by optical density measurements at 570 nm and the values were converted to mg/ml using a factor previously determined.
pH was measured
Emulsification index (EI) was determined by the method described by Fontes et al. (2012). The EI of cell-free samples was determined by adding 1 ml of hexadecane to the same amount of sample, vortex-mixing this mixture for 2 min and leaving to stand for 24 hours. The EI is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm).

2.4 Statistical analysis

The statistical evaluations were performed with the help of the STATISTICA 7.1 software (StatSoft, Inc., Tulsa, OK, USA). Analysis of variance was performed and comparisons between means were performed by the Tukey test with 95% confidence level.

3. Results and Discussion

Fontes et al (2012) optimized media composition for biosurfactant production by *Y. lipolytica* IMUFRJ 50682, However, this medium contains expensive components, such as yeast extract and pure glucose and glycerol. Based on this medium, a substitution of carbon and nitrogen sources were proposed by using wastes: tiger nut fiber (TNF) and corn steep liquor (CSL). Ammonium sulfate was maintained since a nitrogen source is needed for biosurfactant production and this component present in Fontes et al (2012) media is not expensive. The results for cell growth after 48 h of *Y. lipolytica* culture in the different media can be seen in Table 2. It is possible to verify that the cell growth ($[X]_f$) in control medium (CT), that was composed by with yeast extract, ammonium sulfate, glycerol and glucose, was the highest, differing statistically ($p < 0.05$) from final cell concentration of the other media tested.

Table 2: Growth and biosurfactant production parameters for Yarrowia lipolyica cultivated for 48 h in different media

Medium	Parameters		
	$[X]_f$ (g/L)	pH	EI (%)
CT	6.89^a	5.35^a	68.75^a
AmSft	1.63^b	6.99^b	0^c
AmSft + TNF	1.91^b	6.62^c	33.33^b
AmSft + CSL	1.70^b	7.68^b	40.00^b
AmSft + TNF + CSL	2.46^b	7.77^a	63.39^a

CT: control; AmSft: ammonium sulfate; TNF: tiger nut fiber; CSL: corn steep liquor
$[X]_f$: final cell concentration (48 h); pH (48 h); EI: emulsification index after 48 h of culture.
Different letters in the same column differ significantly from each other, by the Tukey test, at a significance level of 5% ($p < 0.05$).

Despite the fact that CSL is composed by nitrogen, organic carbon and vitamins (Table 1), it is possible that the amount used was not sufficient to favor cell growth in CSL + AmSft medium (Table 2). TNF, apparently, also did not favor cell growth (Table 2), even though it presents around 9% of lipids (Table 1) in its composition and *Y. lipolytica* is able to use it as carbon source (Amaral et al., 2006). However, it is possible that this cell concentration in TNF-containing media is a little underestimated due to the migration of *Y. lipolytic* yeast cells to the surface of TNF pellets, which settle to the bottom of the flask, resulting in a low cell

concentration in the liquid part of the medium. When TNF was used with CSL, an increase in $[X]_f$ was noted, but it was not considered statistically significant.

Table 2 also shows the production of biosurfactant after 48 h of culture, detected by the emulsification index, for these same culture media. In the control medium (CT) there was a high emulsification index (60%), corroborating with the results of Fontes et al. (2012), which showed that this medium composition favors the production of biosurfactant. The medium containing only ammonium sulfate (AmSft) did not produce biosurfactant, as expected, since carbon source is needed for biosurfactant production. However, it can be seen that in media containing ammonium sulfate and residues tested separately (AmSft + TNF and AmSft + CSL) presented expressive IE values, but still lower than the CT medium. Even so, this demonstrates the biosurfactant production potential of these wastes. In fact, it was observed that the IE in the medium containing TNF and CSL, in addition to AmSft, was statistically the same to the control ($p > 0.05$). It is therefore suggested that the metabolic pathway of cell growth is deviated for biosurfactant production, probably due to the unbalance of the ideal C/N ratio for cell growth of *Y. lipolytica*. Corn steep liquor had already been tested for biosurfactant production by *Candida lipolytica* in a medium containing animal fat. Reduction of surface tension from 50 to 28 mN/m was found with high emulsification index (Santos et al., 2013). However, this is the first report in literature of the use of TNF waste as carbon source for biosurfactant production.

The pH of the medium after 48 h was around 7.0, for all media, except for the control. However, for medium containing solid residue of tiger nut (TNF) and corn steep liquor the pH differed statistically ($p > 0.05$) from the other media tested (Table 2).

Figure 1 shows the kinetic of biosurfactant production and cell growth for the control medium and the medium with both residues (AmSft + TNF + CSL). With this profile it is possible to see that the residues medium was better than the control as emulsification index was already high (around 60 %) after 24 h of culture, when for CT it was still less than 40%. Therefore, the productivity is higher for AmSft+TNF+CSL medium.

For AmSft+TNF+CSL medium the pH did not undergo major changes and was not controlled during the fermentation, varying from 7.3 to 7.8. It is possible that this behavior contributes to obtain higher yields of biosurfactant, as observed by Rufino et al. (2014). By studying the influence of pH on the production of the biosurfactant by *Y. lipolytica*, Zinjarde and Pant (2002) observed that at pH 8.0, better productivity was obtained. In the control (CT) the pH was statistically different from the other media ($p < 0.05$) and oscillated between values of 2.58 and 5.35 maintaining good biosurfactant production. These results demonstrate the efficiency of yeast *Y. lipolytica* to produce biosurfactant over a wide pH range.

Table 3 shows a cost estimative for the formulation of CT and AmSft+TNF+CSL medium. It was considered the lowest price of Sigma-aldrich US (highest amounts sold) and for TNF it was considered the price for the commercial product tiger nut flour, since TNF is not commercially available yet. However, tiger nut flour is surely more expansive than tiger nut fibre. So, this estimative is not a reality since industrial prices are often lower, but it can be used as comparison purposes. Table 3 depicts that AmSft+TNF+CSL medium is less than one third the cost of CT medium, which shows the great potential of using those residues for biosurfactant production.

Table 3: Prices of media components and costs for preparation of 1 litter of CT and AmSft+TNF+CSL medium

Medium component	Source	Code	U$/kg	CT medium		AmSft+TNF+CSL medium	
				g/L	$/L	g/L	$/L
Ammonium sulfate	Sigma-aldrich US	A4915-50kg	16.98	10.0	0.17	10.0	0.17
Yeast extract	Sigma-aldrich US	92144-25kg	93.60	0.5	0.05	0.0	0.00
Glucose	Sigma-aldrich US	G8270-25kg	9.56	40.0	0.38	0.0	0.00
Glycerol	Sigma-aldrich US	G2289-20L*	48.20	20.0	0.96	0.0	0.00
Tiger nut fibre	https://www.thetigernutcompany.co.uk	TN012**	18.91	0.0	0.00	10.0	0.19
Corn steep liquor	Sigma-aldrich US	C4648-2,5kg	50.80	0.0	0.00	2.5	0.13
Total cost (U$/L)					1.56		0.49

*considering glycerol density of 1.25 g/mL

**As tiger nut fibre is not commercially sold, it was considered the price of tiger nut flour

CT: control; AmSft: ammonium sulfate; TNF: tiger nut fiber; CSL: corn steep liquor

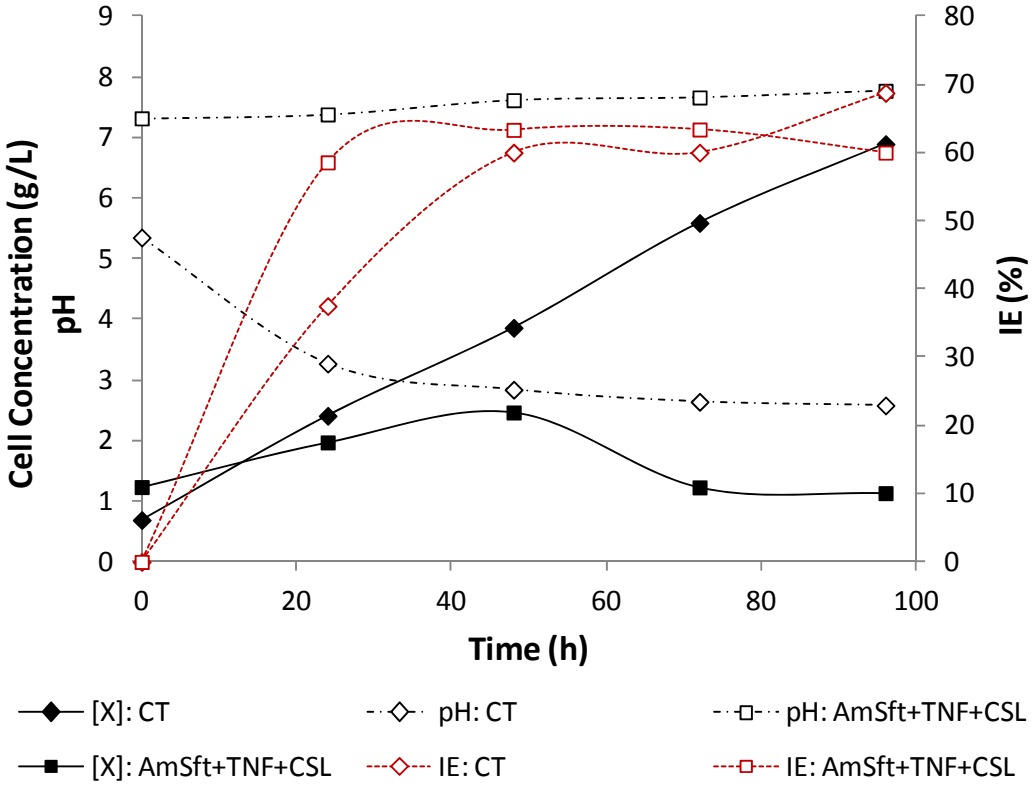

Figure 1: Cell concentration ([X]), pH and biosurfactant production (IE: emulsification index) during time for Yarrowia lipolytica cultivated in CT medium (yeast extract, ammonium sulfate, glycerol and glucose) and AmSft + TNF + CSL medium (Ammonium sulfate, tiger nut fibre and corn steep liquor).

4. Conclusions

Tiger nut fibre (TNF), a by-product from “Horchata de chufa” production, and corn steep liquor (CSL), a waste from starch industry can be valorised for the production of biosurfactant. Each residue separately used with ammonium sulphate was able to increase emulsification index in culture medium of *Y. lipolytica* (33 and 40 %, respectively). Both residues can be used together to substitute yeast extract, glucose and glycerol, producing the same emulsification index (60 %) in the culture medium, in an earlier time, increasing productivity. The cost for the formulation of AmSft+TNF+CSL medium is one third the cost for CT medium.

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