

Full Length Research Paper

## ***Pichia* spp. yeasts from Brazilian industrial wastewaters: Physiological characterization and potential for petroleum hydrocarbon utilization and biosurfactant production**

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Accepted 24 January, 2014

*Pichia* strains isolated from industrial wastewaters were physiologically characterized and tested for their potential for hydrocarbon utilization and biosurfactant production using hydrophobic and hydrophilic substrates. The emulsification index and growth curves were obtained to establish the relation between cell growth and this index. *Pichia anomala* CE009 grew in the range of 18 to 39°C and the optimum was 24°C. The strain was able to grow in NaCl concentrations between 2 and 15%, and pH values between 2 and 12. *Pichia membranaefaciens* CE015 grew at temperatures of 18 to 42°C and the optimum was 30°C. This strain grew in 2 to 10% of NaCl and pH from 2 to 12. Hexadecane, kerosene and diesel oil were used for growth but not for biosurfactant production, while glucose and glycerol were used for growth and biosurfactant production. Biosurfactant was detected during the exponential growth phase, with production peaks of 63% for *P. anomala* CE009 and 58% for *P. membranaefaciens* CE015. This study shows the potential of two *Pichia* strains for bioremediation exploitation under a wide range of environmental conditions.

**Key words:** Emulsifying agents, *Pichia anomala*, *Pichia membranaefaciens*, glycerol.

### INTRODUCTION

Yeasts have emerged as an important group with significant biological relevance and environmentally relevant applications. The great advantage of using yeasts is the generally regarded as safe (GRAS) status that most yeast species present. Organisms with GRAS status are not toxic or pathogenic; a fact that increases the range of possible uses such as for biodegradation and biosurfactant production (Fontes et al., 2012).

Kerosene and diesel oil, composed by fractions of aromatic and aliphatic hydrocarbons, are examples of environmental pollutants (Saratale et al., 2007), while the hexadecane (HXD), present in the aliphatic fraction of

crude oil (Chénier et al., 2003), has been used as a model molecule to study aliphatic hydrocarbon utilization (Schoefs et al., 2004).

Conventional chemical surfactants are organic substances composed of hydrophilic and hydrophobic portions. These compounds are commonly used to separate oily materials from a particular medium because they are able to increase the aqueous solubility of non-aqueous phase liquids (NAPLS) by reducing their interfacial tension at the air-water and water-oil interfaces (Yin et al., 2009). These compounds are costly and pose potential threats to the environment due to their recalcitrance and toxicity.

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Tightening environmental regulations and increasing awareness of the need to protect the ecosystem have resulted in growing interest in biosurfactants as possible alternatives to synthetic ones (Banat et al., 2010). When properly used, biosurfactants are comparable to traditional chemical analogues in terms of performance and offer advantages of low toxicity, high biodegradability, high foaming capability, higher selectivity, specific activity at extreme temperature, pH and salinity, ability to be synthesized from renewable substrates and ecological acceptability (Fontes et al., 2008). Their environmental uses are related principally to the bioremediation of petroleum hydrocarbons in groundwater and soil and the breakdown of hazardous compounds (Coimbra et al., 2009). The addition of biosurfactants increases the availability of long-chain hydrocarbons to microbes and renders them more accessible to microbial enzyme systems for utilization (Khopade et al., 2012a, b).

Although the best known biosurfactants are of bacterial origin, as reported by Abouseoud et al. (2008), Wu et al. (2008), Das et al. (2009), Pansiripat et al. (2010), Abbasi et al. (2012), Eddouaouda et al. (2012), Khopade et al. (2012a, b), Sousa et al. (2012) and Burgos-Díaz et al. (2013), the study of biosurfactant production by yeasts has been growing in importance, with production being reported mainly by the genera *Candida* sp., *Pseudozyma* sp. and *Yarrowia* sp. (Morita et al., 2007, 2008; Ilori et al., 2008; Luna et al., 2009; Batista et al., 2010; Daverey and Pakshirajan, 2010; Luna et al., 2011, 2012; Accorsini et al., 2012; Fontes et al., 2012; Rufino et al., 2012; Chung et al., 2013). *Pichia* spp. are very interesting yeasts from the standpoint of environmental applications (Walker, 2011), but works on biosurfactant production by this genus are still scarce. Indeed, one study with *Pichia anomala* PY1, was done by Thaniyavarn et al. (2008) and another with *Pichia jadinii* by Dziegielewska and Adamczak (2013). Thus, the investigation of the potential of *Pichia* species to produce biosurfactants is an innovative aspect of this study.

The carbon source is a limiting factor in the production costs of biosurfactants (Abouseoud et al., 2008; Das et al., 2009). A possible strategy to reduce these costs is the use of alternative substrates, such as agricultural and industrial wastes. Some examples of residue substrates are different types of used oils, waste from the processing of plants such as molasses and recently glycerol, a residue from the production of biodiesel (Silva et al., 2009; Batista et al., 2010; Dobson et al., 2012; Luna et al., 2012).

Microbial applications are subject to changing environmental conditions, such as salinity, temperature and pH, to which the microorganisms must adapt in order to survive. In this sense, the present work aimed to evaluate the effects of pH, temperature and salinity on growth of *P. anomala* CE009 and *Pichia membranaefaciens* CE015 and to test the potential of these strains for petroleum hydrocarbon utilization and biosurfactant production using hydrophilic and hydrophobic substrates as carbon and

energy sources.

## MATERIALS AND METHODS

### Yeasts

*P. anomala* CE009 and *P. membranaefaciens* CE015 were obtained from the culture collection of the Environmental Microbiology Laboratory of the Biology Department of Federal University of Ceará, Brazil. These strains are maintained at 4°C on potato dextrose agar (PDA) (Oxoid) covered with mineral oil. *P. anomala* CE009 was isolated from a cashew nut processing plant effluent and *P. membranaefaciens* CE015 from an oil refinery effluent, both in Fortaleza, Ceará, Brazil. The strains were previously identified by conventional methods used in yeast taxonomy (Kurtzman and Fell, 1998).

### Organic substrates

Kerosene and diesel oil were supplied by the oil company Petrobras. Glycerol, glucose and hexadecane were provided by Sigma-Aldrich (USA).

### Inoculum standardization

The strains *P. anomala* CE009 and *P. membranaefaciens* CE015 were grown in yeast malt agar (YMA) medium at 25°C for 48 h. Then, three colonies from of each strain were transferred to 250-mL Erlenmeyer flasks containing 50 mL of yeast malt broth (YMB) composed of peptic digest of animal tissue 5.0 g L<sup>-1</sup>, yeast extract 3.0 g L<sup>-1</sup>, malt extract 3.0 g L<sup>-1</sup>, dextrose 10.00 g L<sup>-1</sup>, final pH (at 25 °C) of 6.2 ± 0.2. The flasks containing the strains were incubated in a rotary shaker at 150 rpm at 25°C for 24 h, after which the optical density (OD) of the cultures were adjusted to approximately 0.5 at 600 nm, corresponding to a density of 10<sup>6</sup>-10<sup>7</sup>CFU mL<sup>-1</sup> according to the calibration curve of CFU mL<sup>-1</sup> versus OD.

### Physiological characterization

Aliquots of 1 mL of inoculum of the strains *P. anomala* CE009 and *P. membranaefaciens* CE015 were transferred to flasks containing 5 mL of yeast malt broth (YMB) and incubated at 18, 21, 24, 27, 31, 33, 36, 39, 42 and 45°C for 48 h. The microbial growth at each temperature was measured by OD at a wavelength of 600 nm after 24 and 48 h. The effect of pH on the growth was evaluated by adjusting the pH in YMB by adding sterile 1 M HCl or 1 M NaOH to pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. Aliquots of 1 mL of inoculum of the strains *P. anomala* CE009 and *P. membranaefaciens* CE015 were transferred to flasks containing 5 mL of YMB at different pH values. The cultures were incubated at 25°C for 48 h. The microbial growth at each pH was measured by OD at a wavelength of 600 nm at 24 and 48 h.

The influence of salinity on the growth was evaluated by inoculating of 1 mL of each yeast strain in 5 mL of YMB containing 2, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15 and 16% of NaCl (w/v). The media were then incubated at 25°C for 48 h. The microbial growth was measured by OD at 600 nm wavelength after 24 and 48 h.

### Ability to grow on petroleum derived hydrocarbons

A mineral salt medium containing in (g L<sup>-1</sup>): MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2); CaCl<sub>2</sub> (0.02); K<sub>2</sub>HPO<sub>4</sub> (13.94); KH<sub>2</sub>PO<sub>4</sub> (6.0) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (4.0) was prepared and sterilized at 110°C for 10 min. Afterwards, 0.1%

(v/v) of a micronutrient solution previously sterilized by filtration (0.22  $\mu\text{m}$ , Millipore, USA), was added. The composition of the micronutrient solution per liter was as follows: EDTA (2.5 g),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (10.95 g),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (5.0 g),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (1.54 g),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.392 g),  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  (0.25 g),  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  (0.177 g) (Bushnell and Haas, 1941). This solution was acidified with a few drops of concentrated sulfuric acid, in order to prevent precipitation (Sousa et al., 2012). Finally, substrates n-hexadecane, kerosene and diesel oil, sterilized in a membrane filter (0.22  $\mu\text{m}$ , Millipore), were added aseptically to attain final concentrations of 2% (v/v). Triplicate Erlenmeyer flasks of 250 mL with 50 mL of hexadecane salt medium (HSM), kerosene salt medium (KSM) and oil diesel salt medium (ODSM) were inoculated separately with 1 ml of *P. anomala* CE009 and *P. membranaefaciens* CE015 and incubated at 25°C with shaking speed of 150 rpm for a period of 4 days. To monitor microbial growth, OD of the cultures was measured at 600 nm at intervals of 1 day during 4 days.

### Production of biosurfactant

The same procedure described above for the preparation of HSM, KSM and ODSM was applied to prepare glycerol salt medium (GLYSM) and glucose salt medium (GSM) which also were inoculated separately with equal volumes of *P. anomala* CE009 and of *P. membranaefaciens* CE015. The cultures were incubated at 25°C with shaking speed of 150 rpm for 4 days. Afterwards, the cultures were centrifuged at 10,000 g for 15 min, at 4°C and the emulsification index ( $E_{24}$ ) of the supernatant was measured by adding 2 mL of kerosene and 2 mL of the cell-free broth in a test tube, which was vortexed at high speed for 2 min and allowed to stand for 24 h. After this period, the height of the emulsion layer was measured and the emulsification index ( $E_{24}$ ) was calculated using Equation (1), according to Desai and Banat (1997):

$$EA_{24\%} = \text{HEL/HS} \times 100 \quad (1)$$

Where, HEL is the height of the emulsified layer (cm) and HS is the total height of liquid column (cm). The  $EA_{24}$  index was given as percentage of the emulsified layer height (cm) divided by the total height of the liquid column (cm).

### Statistical analysis

Statistical analysis was performed by one-way repeated measure ANOVA in association with the post-hoc Tukey test and paired-samples t-test using Prism 5 (GraphPad Software). A  $p < 0.05$  was considered significant. All experiments were performed in triplicate with at least two repetitions.

## RESULTS

### Physiological characterization

Figure 1 shows that the optimum growth conditions were reached at a temperature range of 25-30°C, pH 3.0-4.0 and 2-3% of NaCl for *P. anomala* CE009 and *P. membranaefaciens* CE015. The results also showed that the growth of *Pichia* species decreased under extreme conditions. The growth of the *P. membranaefaciens* CE015 increased until 42°C, while *P. anomala* CE009 increased until 39°C. However, that strain grew up to a NaCl concentration of 15% while *P. membranaefaciens* CE015 only grew up to 10% of that salt, indicating that *P. anomala* CE 009 shows better adaptation to high concentration of NaCl than *P. membranaefaciens* CE015. Both strains grew

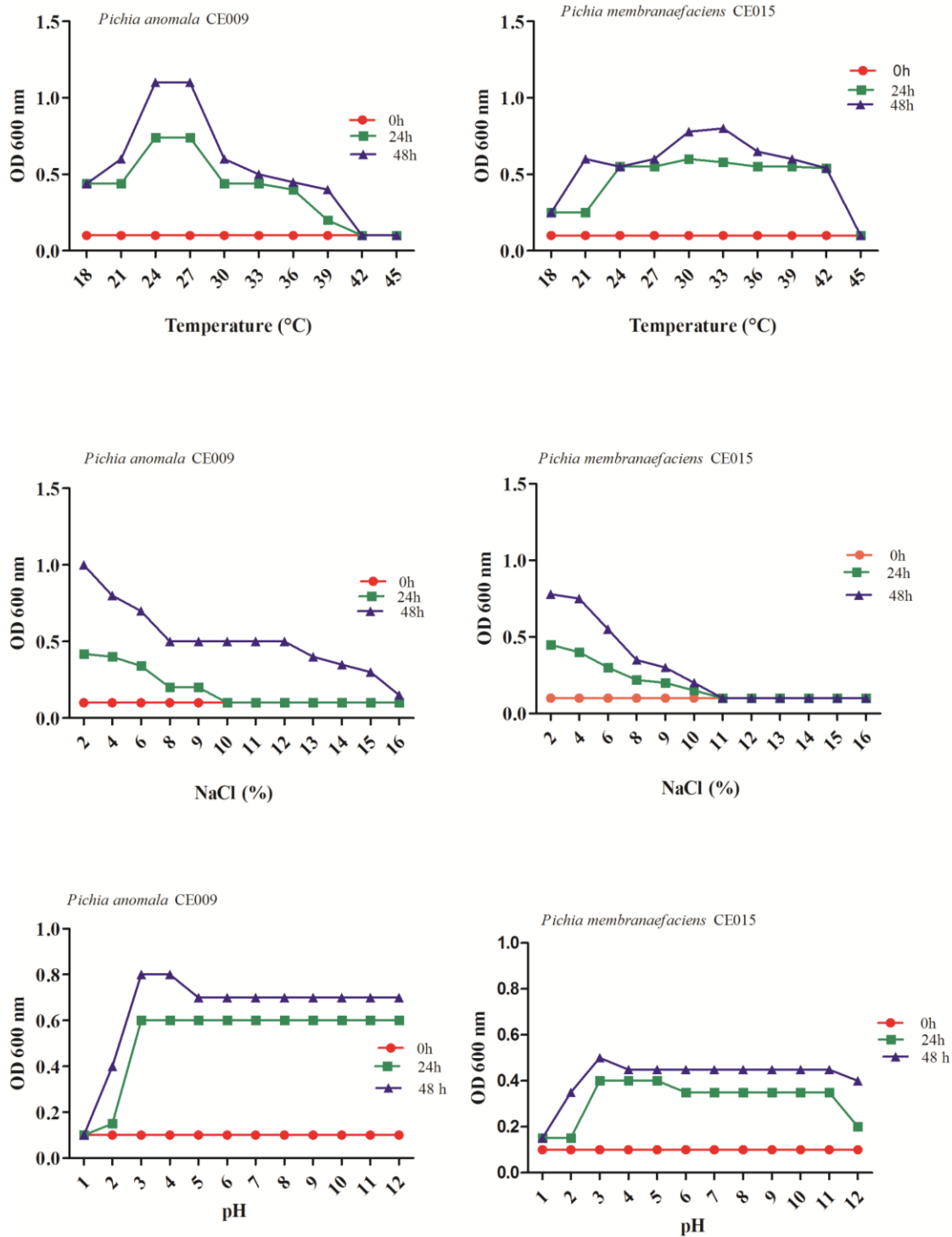
at extreme pH values of 2 and 12 but, the growth of *Pichia* species differed in their relative sensitivity to the factors evaluated. In general, *P. anomala* CE009 showed significantly higher cell concentration ( $p < 0.05$ ) than *P. membranaefaciens* CE015 when subjected to the same conditions (Figure 1).

Growth dynamics of two *Pichia* strains on petroleum hydrocarbons (kerosene and diesel oil) and aliphatic hydrocarbon n-hexadecane did not reveal a lag phase and the utilization of hydrocarbon substrates was shown by increase in the number of cells (Figure 2). Hexadecane was used similarly for growth of both *P. anomala* CE009 and *P. membranaefaciens* CE015. The results of kerosene and diesel oil utilization varied significantly between the strains ( $p < 0.05$ ). Kerosene was more efficiently used by *P. membranaefaciens* CE015 in comparison to *P. anomala* CE009, which was more efficient in using diesel oil as the carbon and energy source.

Tests carried out with the *P. anomala* CE009 and *P. membranaefaciens* CE015 using kerosene, diesel oil and hexadecane as substrate showed absence of emulsifying activity or unstable emulsification. However, the utilization of the glucose and glycerol by two *Pichia* strains was accompanied by an increase in cell density, with concomitant surfactant activity. The growth dynamics of *P. anomala* CE009 did not reveal a lag phase and the biosurfactant production started at the exponential growth phase (Figure 3A). The growth dynamics of *P. membranaefaciens* CE015 revealed a slight lag phase, but biosurfactant production was not delayed (Figure 3B). For *P. anomala* CE009, the emulsification indexes ( $E_{24}$ ) ranged from 48 to 58% on glucose and from 39 to 63% on glycerol (Figure 3A). For *P. membranaefaciens* CE015, these ranges were from 38 to 51% on glucose and 40 to 58% on glycerol (Figure 3B). Thus, the highest emulsification indexes for *P. anomala* CE009 were 58 and 63% on glucose and glycerol, respectively, and 51 and 58% for *P. membranaefaciens* CE015 on the same substrates. These values were detected at the end of the exponential growth phase ( $t = 2$  days) and from the fourth day onward a gradual decrease in the two isolates population was observed (Figure 3A and B). The emulsification indexes were higher with glycerol than with glucose for both yeast strains and the emulsification indexes of *P. anomala* CE009 were higher than those of *P. membranaefaciens* CE015 on both substrates.

## DISCUSSION

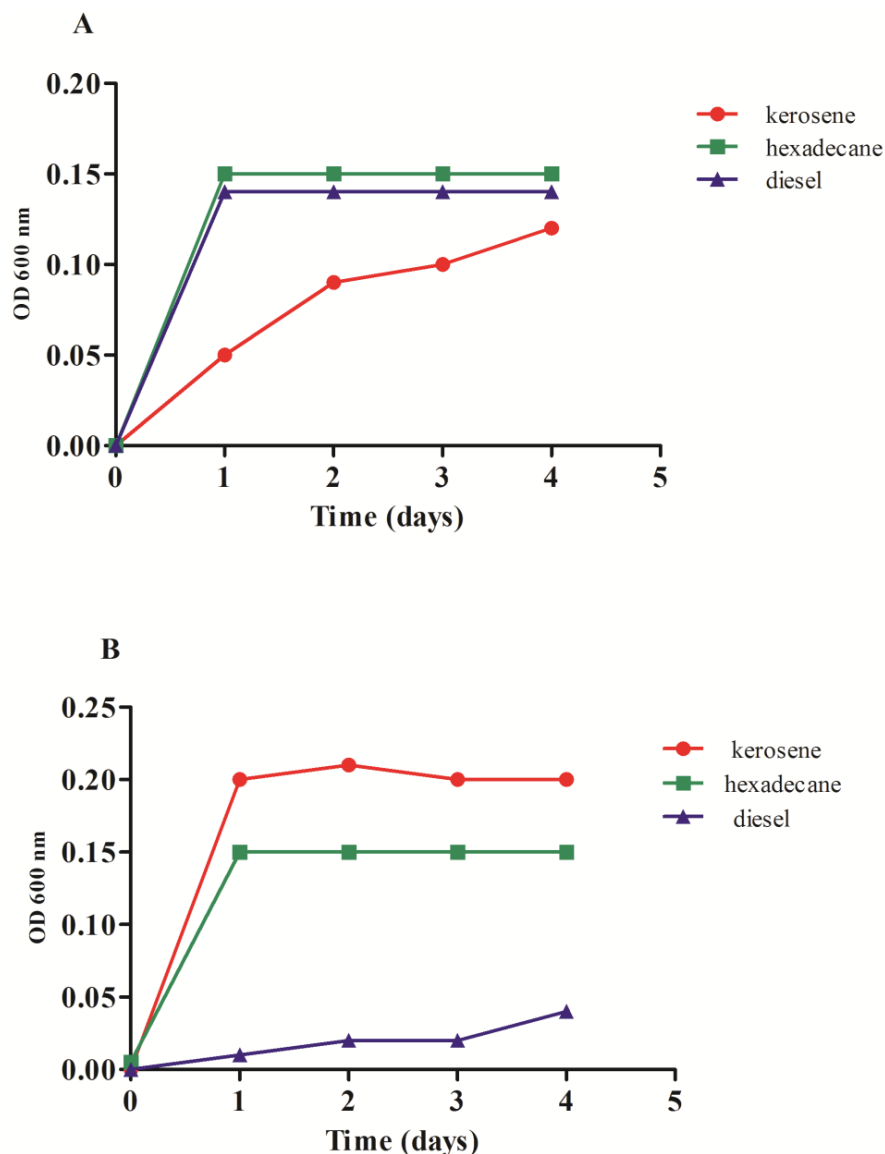
The growth of *P. anomala* CE009 and *P. membranaefaciens* CE015 under extreme values of pH (2 and 12), salinity (2 and 15%) and temperature (18 and 42°C) showed the wide spectrum of plasticity of these strains to environmental conditions. From an ecological point of view, this plasticity can be a tool for survival under the environmental conditions prevalent in the cashew nut processing and oil refinery effluents.



**Figure 1.** Effects of temperature salinity and pH on growth of *P. anomala* CE009 and *P. membranaefaciens* CE015 in malt broth medium at 0, 24 and 48 h. Each point represents the average value obtained with three independent experiments.

*P. anomala* CE009 and *P. membranaefaciens* CE015 were categorized as thermotolerant, on the basis of their growth below 20°C, and up to high temperatures, such as 37-48°C (Arthur and Watson, 1976; Limtong et al., 2005).

Thermotolerant yeasts have the advantage that they can be cultured under conditions where other microorganisms cannot grow, which reduces the risk of contamination. Thermotolerant enzymes made by yeasts may possess



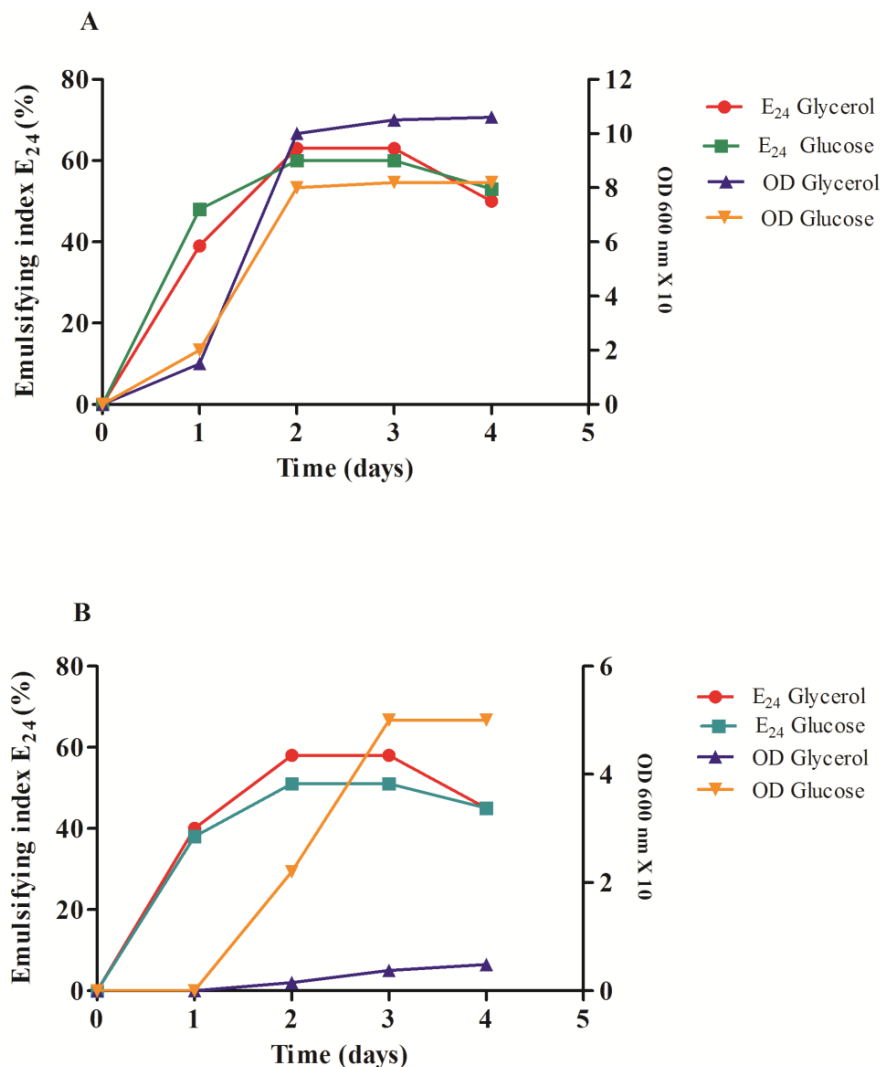
**Figure 2.** Growth profiles of *P. anomala* CE009 (A) and *P. membranaefaciens* CE015 (B) in mineral salts medium containing 2% of kerosene, diesel oil and hexadecane as the carbon and energy sources. Incubation was done at 26°C with stirring at 150 rpm. Each point represents the average value obtained with three independent experiments.

special advantages because of their eukaryotic nature, when compared with enzymes from thermotolerant bacteria or archaea (Takashima et al., 2009).

In this work, *P. anomala* CE009 and *P. membranaefaciens* CE015 were able to grow in salt concentrations of 10-15% and were classified as halotolerant. High salinity poses osmotic stress and specific ion toxicity for yeast cells (Ren et al., 2012) and salt-tolerance is a strategy of cellular osmotic adaptations at the physiological and molecular level to combat fluctuating salinity (Plemenitaš et al., 2008). Jadhav et al. (2010) emphasized that halotolerant microorganisms are known to be potential sources of extracellular enzymes with novel properties, useful for diverse industrial applications.

Yeasts are usually acid tolerant and grow at  $\text{pH} \leq 4.0$  (Gross and Robbins, 2000), but according to Zvyagilskaya and Persson (2004),  $\text{pH} 10.0$  is the upper pH limit for yeast growth and alkalization of the external environment represents a stress situation for most yeast strains (Serrano et al., 2006). Thus, the high alkali tolerance exhibited by *P. anomala* CE009 and *P. membranaefaciens* CE015 makes these *Pichia* strains promising for applications under extreme alkaline environmental conditions.

*P. anomala* CE009 showed higher cell concentration than *P. membranaefaciens* CE015 under adverse conditions of temperature, pH and salinity, suggesting the better potential of this strain for bioremediation applications in extreme environments.



**Figure 3.** Population dynamics (OD) and emulsification index (E<sub>24</sub>) during cultivation of yeasts strains *P. anomala* CE009 (A) and *P. membranaefaciens* CE015 (B) on glycerol and glucose. Incubation was done at 26°C with shaking at 150 rpm. Glycerol and glucose were supplied at 2% (v/v). Values are averages of three replicate determinations.

The growth on hexadecane, kerosene and diesel oil indicated the ability of *P. anomala* CE009 and *P. membranaefaciens* CE015 for utilization of these petroleum hydrocarbons as carbon and energy source. The absence of lag phase in growth curves of *P. anomala* CE009 and *P. membranaefaciens* CE015 was suggestive of the pre-adaptation of these strains to hydrocarbon substrates. For *P. membranaefaciens* CE015 that originated from oil refinery effluent, this result was expected but for *P. anomala* CE009, which was isolated from a cashew nut processing plant effluent, it was surprising. The improvement of the cashew nut is one of the industrial activities that have larger importance, principally economic in States North-east of the Brazilian. In view of that intense productive activity, those industries generates lots of liquid effluents,

whose characteristics are not still well known due to the small amount of scientific works accomplished in the area (Pimentel et al., 2009). However, Rajeswari et al. (2011) reported that the cashew nut shell liquid (CNSL), a phenolic oily liquid is the most abundant by-product of the cashew nut processing which may explain the behavior of *P. anomala* CE009 front to hydrophobic hydrocarbons evaluated in this study.

The maximum value of emulsification index registered was 63% from *P. anomala* CE009 cultivated in glycerol over a period of 2 days. Fontes et al. (2012) reported that *Yarrowia lipolytica* was able to grow and produce biosurfactant on crude glycerol, achieving maximum emulsification index of 70.2%. The fact that biosurfactant production has been higher in the medium containing crude

glycerol than in pure glycerol can be attributed the presence of fatty acids (Fontes et al., 2012), although Yang et al. (2012) have discussed that pollutants in crude glycerol, can inhibit cell growth and result in lower product yields (when compared with commercial glycerol under the same culture conditions). Chandran and Das (2010) showed that emulsification index of the Tween 80 was 67% and the present study showed that the value to *P. anomala* CE009 was quite higher than the chemical commercial surfactant.

The profile of growth curves of *P. anomala* CE009 and *P. membranaefaciens* CE015 on hydrophilic substrates (Figure 3), confirmed that glycerol usually serves as a substitute for glucose (Bognolo, 1999). To Ilori et al. (2008), the exact reasons why some microorganisms produce biosurfactants are still not clear but according Salihu et al. (2009), the carbon source is very important in the production of these substances and includes carbohydrates, hydrocarbons and vegetable oils. To Abasi et al. (2012), the microorganisms differ in their capacity to produce biosurfactants, some use as substrates only carbohydrates, others only hydrocarbons, and still others consume several substrates, in combination or separately. In this work, while the hydrophobic substrates were inefficient for biosurfactant production the hydrophilic substrates (glucose and glycerol) were used for this objective by *P. anomala* CE009 and *P. membranaefaciens* CE015. These observations are in accordance with data in the literature on the synthesis of biosurfactants by yeasts strains from hydrophilic substrates such as glucose (Dziegielewska and Adamczak, 2013) and sugarcane molasses (Takahashi et al., 2011). Wu et al. (2008) reported similar results for *Pseudomonas aeruginosa* EM1, while, Queiroga et al. (2003) showed that *Bacillus subtilis* C9 produced high yields of biosurfactant using a soluble carbohydrate substrate, while a hydrocarbon substrate inhibited the production of this substance. In contrast, Nitschke et al. (2005) described that hydrophilic carbon sources such as glycerol present lower yield of biosurfactants as compared to hydrophobic sources such as soybean oil.

Although, most biosurfactants are considered secondary metabolites (Singh and Cameotra, 2004), in this study, the emulsifying index values indicate that the biosurfactant biosynthesis occurred predominantly during the exponential growth phase, suggesting that the biosurfactant is produced as primary metabolite accompanying cellular biomass formation (Cunha et al., 2004). For *P. anomala* CE009 and *P. membranaefaciens* CE015 the biosurfactant production ceased when growth stopped and population density started decreasing (Figure 3) probably due to the production of secondary metabolites that could interfere with emulsion formation. Khopade et al. (2012a) also reported that a biomulsifier synthesized by a strain of *Bacillus* sp. was produced as a primary metabolite accompanying a cellular biomass formation. In contrast, Amaral et al. (2006) and Accorsini et al. (2012) reported

that the most biosurfactants are usually produced when the cultures reach the stationary phase of growth.

This affirmation is corroborated by Ilori et al. (2008) which showed that biosurfactant synthesis by the *Saccharomyces cerevisiae* and *Candida albicans* was maximal for cells in stationary/death phase and in accordance with previously reported data by Rodrigues et al. (2006) on kinetic study of biosurfactant production by *Lactobacillus* strains. It was found that the best biosurfactant-producing *Pichia* species was *P. anomala* CE009 with an  $E_{24}$  of 63%. *P. anomala* PY1 was described by Thaniyavarn et al. (2008) as biosurfactant producer by using soybean oil as carbon source. Except for this report, other *Pichia* species such as *P. membranaefaciens* has not been reported previously for biosurfactant production which highlights the innovative aspect of this work.

On the other hand, according to Dobson et al. (2012), although the current price of glucose is comparable to that of crude glycerol (US \$0.21-0.23/lb), strong price fluctuation has been observed in the last 15 years, with prices reaching US \$0.40/lb at the beginning of 2010. In addition, recent surge in biodiesel production has led to increased accumulation of glycerol as byproduct of this industry. As consequence, the price of crude glycerol is continuously decreasing and a negative value will be attributed to crude glycerol in the future, which will increase the interest to use it as a biological feedstock for the production of economically value-added products, as the biosurfactants (Coombs, 2007; Pagliaro et al., 2009), but few publications present the possibility to utilize waste glycerol for biosurfactant synthesis (Morita et al., 2007). Although this study has utilized glycerol commercial the results indicate the potential of *P. anomala* CE009 and *P. membranaefaciens* CE015, particularly the first strain, for biosurfactant production from glycerol.

## Conclusion

*P. anomala* CE009 and *P. membranaefaciens* CE015 represent a potential, still unexploited, for studies on biotransformation of toxic pollutants and biosurfactant production on co-substrates such as sugar and glycerol.

## ACKNOWLEDGEMENTS

The authors wish to thank Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and Funcap (Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico) for financial support.

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