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Utilization of glycerin byproduct derived from soybean oil biodiesel as a carbon source for heterologous protein production in *Pichia pastoris*

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HIGHLIGHTS

- Up to 40,000 tons per year of glycerin will be produced from biodiesel by 2020.
- This study evaluated 11 glycerin samples for *Pichia pastoris* growth.
- For the first time glycerin from soybean oil was utilized for *Pichia pastoris* growth.
- Crude glycerin samples prepared with sodium hydroxide resulted in 1.5 higher OD.
- All together results highlight an important application for glycerin from soybean oil.

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ABSTRACT

Crude glycerol, also known as glycerin, is the main byproduct of the biodiesel industry. It has been estimated that up to 40,000 tons of glycerin will be produced each year by 2020. This study evaluated the value-added use of crude glycerol derived from soybean biodiesel preparation as a carbon source for heterologous protein production using the yeast *Pichia pastoris*. Eleven glycerin samples were obtained by methanolysis of soybean oil using different acids or bases as catalysts. Cell growth experiments showed that crude glycerol containing either potassium or sodium hydroxide resulted in 1.5–2 times higher final cell densities when compared to glycerol P.A. Finally, crude glycerol containing sodium hydroxide was successfully utilized for constitutive heterologous α-amylase production in *P. pastoris*. This study demonstrated that crude glycerol without any purification steps may be directly used as carbon source for protein production in *P. pastoris*.

1. Introduction

The search for alternative fuels has led to an increase in biodiesel production reaching up to 20 billion liters per year in just over a decade (Emerging Markets Online, 2008). During the biodiesel production process, triglycerides originating from vegetable oils such as soybean, sunflower, canola or rapeseed oil are transesterified with an alcohol, usually methanol, leading to a mixture of fatty acid mono-esters, known as biodiesel, and crude glycerol, typically known as glycerin. Generally, biodiesel production generates approximately 10% by weight of crude glycerol (Johnson and Taconi, 2007). It has been estimated that by 2020, up to 40,000 tons per year of crude glycerol will be produced solely from biodiesel synthesis (Bauer and Hulteberg, 2013).

As crude glycerol is considered a byproduct of the biodiesel industry, its utilization has been considered for the microbial synthesis of a variety of chemicals as has been recently reviewed (Almeida et al., 2012; Liu et al., 2012; Yang et al., 2012). Nevertheless, crude glycerol contains several impurities that make its disposal difficult and may impair microbial growth. For example, the salts, methanol and fatty acids present in crude glycerol were reported to inhibit *Clostridium pasteurianum* growth (Venkataramanan et al., 2012). In this case, the fermentation behavior of the bacteria was only restored when fatty acids were removed by acid precipitation. A similar conclusion was made in another study which evaluated biodiesel-derived glycerin for the microbial growth and production of 1,3-propanediol by *Citrobacter freundii* (Anand and Saxena, 2012). Again, glycerin was found to significantly inhibit bacterial growth because of the high concentrations of free fatty acids and free methyl esters. Yeasts have also been considered as biocatalysts for the production of several compounds such as bioethanol (Liu et al., 2012; Choi et al., 2011), lipids (Duarte et al., 2013; Galafassi et al., 2012), carotenoids (Cutzu et al., 2013)

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and heterologous proteins (Celik et al., 2008; Tang et al., 2009) using glycerin as carbon source. Among the yeast species used, *Pichia pastoris* is known for its ability to produce high levels of recombinant proteins at industrial scale using glycerol as the substrate for fast growth in order to obtain high cell densities (Hohenblum et al., 2004). This ability to consume glycerol can be explained by the presence of four genes coding for glycerol transporters which are responsible for high, specific glycerol uptake rates (Mattanovich et al., 2009). Moreover, *P. pastoris* is easy to manipulate and has the ability to perform complex post-translational modifications (Cereghino et al., 2002). Finally, *P. pastoris* is able to tolerate and use methanol, the most common alcohol used during biodiesel synthesis, as carbon source (Meneghetti et al., 2006).

According to the National Petroleum Agency, ANP, in Brazil about 75% of the biodiesel produced in 2013 is derived from soybean oil as a raw material. Therefore, several crude glycerol samples prepared from this source were evaluated as a carbon source for *P. pastoris* growth. Moreover the effects of a selected glycerin sample were assessed in the production of heterologous α-amylase expression in *P. pastoris*. Growth experiments have shown for the first time that glycerin prepared with either potassium or sodium hydroxide resulted in 1.5–2 times higher final OD when compared with cells grown in glycerol P.A. Finally, crude glycerol contaminating sodium hydroxide was successfully utilized for constitutive heterologous α-amylase production.

2. Methods

2.1. Strain and media

P. pastoris X-33 (Invitrogen, USA) was used in this study. Cells were typically grown in YPD plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar) incubated at 28 °C for 4 days. YPG medium (1% yeast extract, 2% peptone supplemented with either 1% or 2% glycerol) was used for glycerin screening and heterologous protein production.

2.2. Glycerin samples derived from biodiesel synthesis

Samples of glycerin were prepared by soybean oil methanolysis using different catalysts and purification methods (Table 1). In a typical procedure, 500 mL of soy oil, 200 mL of methanol and 5 g of catalyst were added to a round bottom flask and kept under magnetic stirring and reflux for 2 h. In sequence, glycerin was separated from the resulting mixture by decantation using a separating funnel. In some cases, the obtained glycerin was neutralized by adding a neutralizing agent of choice until a neutral pH was obtained. For some samples, either neutralized or non-neutralized, methanol was removed via distillation under reduced pressure at 40 °C. Commercial glycerol P.A. (Sigma–Aldrich) was used as control during growth experiments.

2.3. Effect of P. pastoris cell growth in crude glycerol samples

P. pastoris cell growth was tested in deep well plates using 1% glycerin from different samples (Table 1) in addition to glycerol P.A. One milliliter of YP medium was mixed with each different glycerin sample, and cells were inoculated to an initial density (OD_{600}) of 0.3. For each glycerin sample tested, YPG medium without any cells was used as blank for OD_{600} measurements since each sample has a peculiar aspect depending on its preparation. The surface of the deep well plates was sealed with an adhesive and punctured with a needle for aeration. Plates were placed under stirring (200 rpm) in a rotatory shaker at 28 °C for 96 h. Cell growth (OD_{600}) was analyzed by spectrophotometry (spectrophotometer Biochrom Libra S2/S2B) every 24 h. Growth tests were performed in duplicate.

Table 1
Glycerin samples obtained from the methanolysis of soybean oil using different catalysts and purification procedures.

Sample	Catalyst	Neutralizing reagent	Methanol
A1	H_2SO_4	*	−
A2	HCl	*	−
B	NaOH	*	−
BN1	KOH	$C_6H_8O_7$	−
BN2	$NaHCO_3$	HCl	−
BN3	$NaHCO_3$	H_2SO_4	−
BNM1	KOH	$C_6H_8O_7$	+
BNM2	NaOH	H_3PO_4	+
BNM3	KOH	H_3PO_4	+
BM1	KOH	*	+
BM2	NaOH	*	+

(*) Glycerin was not neutralized; (−) methanol was removed; (+) methanol was not removed.

2.4. P. pastoris growth profile and glycerin utilization rate

P. pastoris growth curves in the presence of 2% glycerin (YPG medium) selected from screening was performed in 1 L baffled Erlenmeyer flasks containing 100 mL YPD medium. A pre-inoculum was inoculated to an initial OD_{600} of 0.2. Flasks were left under stirring (200 rpm) in a shaker at 28 °C for a period of 47 h. At intervals of 3 h, samples were collected to analyze cell density. The test was performed in triplicate.

2.5. Glycerol and methanol quantification

Glycerol and methanol analyses were performed by HPLC (High Performance Liquid Chromatography) Shimadzu using Aminex HPX-87H Column (300 × 7.8 mm) BIO-RAD coupled to a refractive index detector, RID-10A. A sulfuric acid solution (5 mM) was used as the mobile phase at a flow rate of 0.6 mL/min. Column temperature was set to 50 °C. Glycerol samples were diluted to 10% (v/v) in Milli-Q water and filtered through 0.22 μm filters (Millex®). A standard curve for glycerol P.A. and methanol was used for sample quantification.

2.6. Impurity analysis

Total impurities were considered as remaining free fatty acids, monoacylglycerols and biodiesel present in glycerin samples. Analysis was carried out using CTO-20A HPLC equipment (Shimadzu, Tokyo, Japan) with an ultraviolet detector (UV) at 205 nm. All chromatograms were generated using LabSolutions software (Shimadzu, Japan). A single Shim-Pack VP-ODS C18 reversed-phase column (250 × 4.6 mm, 5 μm) was used for the methodology previously described (Carvalho et al., 2012).

Impurities were quantified using soybean oil as the internal standard. Desired sample amounts of glycerin (ca. 0.05 g) and soybean oil (ca. 0.05 g) were dissolved in 2-isopropyl alcohol-n-hexane 5:4 (v/v, ca. 2 g, used as mobile phase). The sum of impurities peak areas where compared with the sum of soybean oil peak areas, whose mass is well-known. Then, the mass of the impurities could be calculated and finally divided by the mass of glycerin samples.

2.7. Constitutive α-amylase production in bioreactor

Construction of the *P. pastoris* strain with an integrated copy of the α-amylase gene from *Bacillus subtilis* under the control of the

Table 2
Glycerin, soybean oil and 2-propanol–hexane 5:4 (v/v)[a] masses used to prepare the samples for HPLC impurity analyses.

Sample	Glycerin (g)	Soybean oil (g)	Solvent (g)	Impurities (%)
Glycerol P.A	0.0593	0.0520	2.0171	4.0
BM1	0.0575	0.0664	2.0066	7.7
BNM1	0.0605	0.0491	2.0094	4.7
B	0.0511	0.0541	2.0143	7.7

[a] Same solvent mixture used as mobile phase.

3-phosphoglycerate kinase promoter (pPGK) has been described elsewhere (de Almeida et al., 2005). Glycerin utilization for growth and recombinant protein production was evaluated in a 2 L BioFlo® 115 (New Brunwick) fermenter. Fermentation media was composed of YPG (2% glycerin). Fermentations were carried out in duplicate for 30 h with the pH controlled with NaOH and dissolved oxygen above 30%.

2.8. Enzymatic activity

Amylase activity was determined as previously described (Marco et al., 1996). In brief, activities on the culture supernatant were assayed using 0.5 M sodium acetate (pH 6.0) and 0.5% soluble starch. After 30 min of incubation at 40 °C, the reaction was quenched by adding 1.0 M acetic acid. Iodine reagent was added to determine dextrinizing activity. One unit of dextrinizing activity was defined as the amount of protein necessary to hydrolyze 0.1 mg starch/min. Protein concentration was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used to determine the standard curve.

3. Results and discussion

3.1. Glycerin samples preparation

In this work, 11 glycerin samples derived from different catalyst and purification treatments have been tested (Table 1). Samples were named according to the catalyst used (A for acid, B for basic) and N refers to samples that were neutralized after the transesterification process. As mentioned previously, methanol was utilized in all transesterification reactions but for some samples, as indicated by confirmed (−), the alcohol was evaporated. Samples where methanol was not evaporated are represented by (+).

Fatty impurities (fatty acids, monoacylglycerols and methylesters) were quantified and the obtained values are shown by percentage in Table 2. It is worth noticing that samples BM1 and B are the most impure. Moreover both samples have not been run through a neutralizing process after the transesterification reaction.

3.2. P. pastoris growth in different glycerin samples

The effects on *P. pastoris* cell growth of the 11 glycerin samples described in Table 1 was assessed using glycerol P.A. as a control. As shown in Fig. 1, four glycerin samples (A1, BN1, BN2 BN3 and BNM2) showed an inhibitory effect on cell growth when compared to *P. pastoris* grown in glycerol P.A. This is probably related to the presence of salts. Salts such as NaCl, KCl and sulfonated compounds such as K_2SO_4 have also been shown to inhibit growth of other microorganisms such as *Paracoccus denitrificans* and *Cupriavidus necator* (Mothes et al., 2007) and *Clostridium pasteurianum* (Venkataramanan et al., 2012).

Conversely, samples B, BNM1 and BM1 resulted in about 1.5 higher optical densities (Fig. 1) as compared to the control. From this initial screening, three glycerin samples (B, BNM1 and BM1) were chosen for the determination of the growth profile and the glycerol utilization rate (Fig. 2A and B).

Sample BNM1 yielded the same growth profile as glycerol P.A. while samples BM1 and B resulted in 2.2 and 1.7 higher final OD, respectively (Fig. 2A). Since all samples started with the same glycerol concentration (Table 3) higher biomass obtained by using BM1 and B may be explained by impurities, here quantified as remaining free fatty acids, monoacylglycerols and biodiesel present in the glycerin samples. In fact, a reduction in those impurities was observed at the end of growth experiments (Fig. 3). In another study, glycerol layer originated from soybean biodiesel was composed of 13% fat (Thompson and He, 2006), thus corroborating with the hypothesis that the yeast is using it as a carbon source for biomass production.

Moreover, when using samples B and BM1 the consumption of methanol during growth was observed, which could also have contributed to the higher final OD observed (Table 3). Together, these results show that the utilization of *P. pastoris* as a biocatalyst for crude glycerol conversion is advantageous since it is not inhibited by free fatty acids, and also because it uses methanol as carbon source. Nevertheless, the glycerol utilization rate was the fastest for media containing glycerol P.A. (2.10 g/L·h^{-1}) as opposed to a previous study where pure glycerol had the slowest glycerol consumption rate (Celik et al., 2008). Samples BM1 and B had about

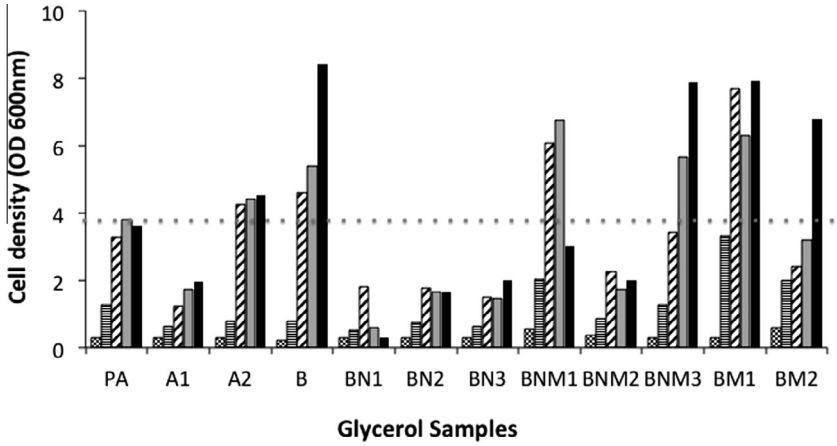

Fig. 1. *P. pastoris* cell growth using different crude glycerol samples as carbon source. Bars represent different time points: 0 h (dotted), 24 h (horizontal line), 48 h (stripped) 72 h (grey) and 96 h (black). Tests were performed in duplicate with standard deviation lower than 10% dotted line indicate maximum cell density obtained when *P. pastoris* was grown using glycerol P.A. as substrate.

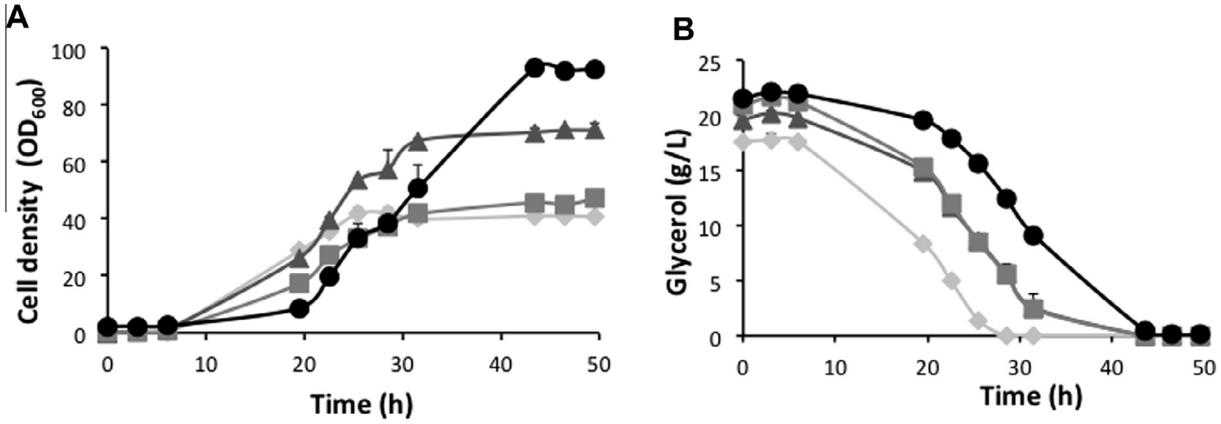

Fig. 2. *P. pastoris* growth profile (A) and glycerol utilization (B) using BNM1 (square), BM1 (circle), B (triangle) and glycerol, P.A (diamond) as carbon source. Growth experiments were performed in triplicate and the standard deviation is indicated in the figure.

Table 3
Initial and final concentrations of glycerol, methanol and impurities measured during aerobic growth of *P. pastoris* using different glycerol samples.

Sample	Initial glycerol concentration (g/L^{-1})	Glycerol concentration $t = 50$ h (g/L^{-1})	Initial methanol concentration (g/L^{-1})	Methanol concentration $t = 50$ h (g/L^{-1})	Initial Impurities in crude glycerol (%)	Impurities $t = 50$ (%)
PA	17.65	0	–	–	4.1	4.4
B	19.65	0	1.18	0.2	7.7	6.1
BM1	21.61	0	27.16	23.55	7.7	6.0
BNM1	20.90	0	17.06	9.96	4.7	5.0

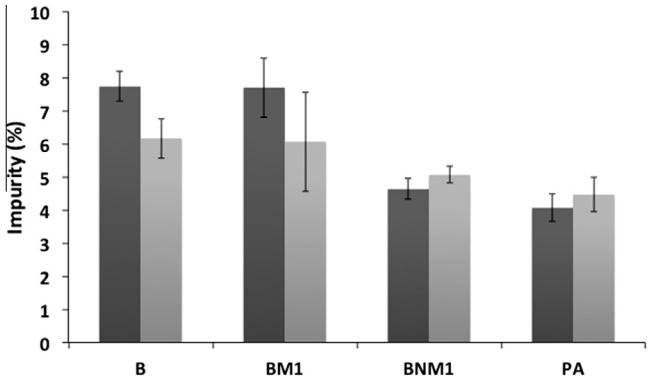

Fig. 3. Total impurities detected in glycerol samples at time zero (dark grey) and 50 h (light grey) of *Pichia pastoris* aerobic growth. The impurities in glycerol PA come from the soybean oil use as internal standard.

half of the glycerol utilization rate at 1.05 $g/L{\cdot}h^{-1}$ (Fig. 2B). Finally, *P. pastoris* growing on BM1 showed the slowest utilization rate of glycerol at 0.66 $g/L{\cdot}h^{-1}$ (Fig. 2B).

It has been reported that the oil type and catalyst utilized for biodiesel production may generate crude glycerol with a variety of impurities such as salts, heavy metals, fatty acids and methanol (Chatzifragkou and Papanikolaou, 2012). Since the purification process is costly, utilization of glycerin for production of high value added products are advantageous and have been considered for a variety of compounds. Nevertheless, microorganisms are known to be growth inhibited when using crude glycerol (Venkataramanan et al., 2012; Anand and Saxena, 2012).

It has been shown that impurities, such as fatty acids with a higher degree of unsaturation, present in crude glycerol had an inhibitory effect on cell growth of the bacterium *C. pasteurianum* (Venkataramanan et al., 2012). Conversely, some studies have shown that yeasts seem to better stand the presence of inhibitory compounds in crude glycerol. For example, ethanol production from *Pachysolen tannophilus* was not affected by any of the three tested crude glycerol batches derived from rapeseed oil. (Liu et al., 2012). In other studies, yeasts isolated from soil (Choi et al., 2011) and Brazilian biomes (Duarte et al., 2013) were able to utilize crude glycerol for the production of ethanol and lipids, respectively. *P. pastoris* has also been utilized for crude glycerin conversion into recombinant proteins (Celik et al., 2008; Tang et al., 2009).

3.3. Impurity analysis

For each sample of crude glycerol utilized in aerobic growth profile determination (Fig. 2), total impurities were quantified (Table 2, Fig. 3). As discussed previously, increased final biomass was attributed to the utilization of free fatty acids, monoacylglycerols and biodiesel. Indeed, samples B and BM1 showed higher concentration of fatty materials when compared with the samples BNM1 and glycerol P.A. In Fig. 3 also shows a certain amount of impurities for the glycerol P.A. As described before, impurities were quantified using soybean oil as internal standard for HPLC analysis. Since the mass of soybean oil and peak areas in the spectrum are well known, by comparison the amount of impurities can be calculated. Therefore, the impurities in glycerol P.A. can only be associated to the internal standard, since the glycerol P.A. is pure.

3.4. Constitutive α-amylase production using crude glycerol

In order to test the effects of glycerin on protein expression, *P. pastoris* transformed with an integrative copy of α-amylase from *Bacillus subtillis* (de Almeida et al., 2005) was utilized for batch fermentations using crude glycerol sample B. This was chosen since it gave a higher final biomass when compared to glycerol P.A. and its glycerol utilization rate was not as slow as for sample BM1. In Fig. 4 shows the fermentation profile of this strain in which the production of recombinant protein was concomitant to biomass

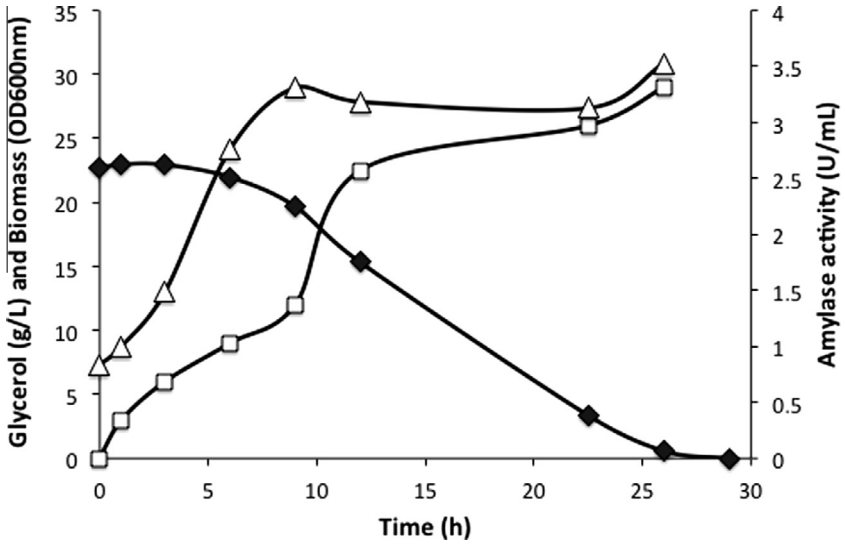

Fig. 4. Fermentation profile of *P. pastoris* using crude glycerol B as carbon source (diamond) and its main products; growth (square) and constitutive α-amylase from *B. subtilis* production (triangle). Fermentations were performed in duplicate and figure show average with less than 10% difference.

formation since a constitutive promoter, pPGK, was utilized. Glycerol was entirely consumed from the glycerin in about 30 h: less than when the yeast was grown in shake flasks. The oxygen transfer rate has been shown to play an essential role in other yeasts growing on glycerin (Liu et al., 2012). Since *P. pastoris* also has a high demand on dissolved oxygen, growth in bioreactors allowed a higher transfer rate and therefore faster utilization of carbon source. Thus, this result demonstrates the potential application of *P. pastoris* in converting glycerin derived from soybean oil to value added products even without any process optimization.

Other studies have shown utilization of glycerin for heterologous protein production. Several glycerin preparations were tested for *P. pastoris* growth and recombinant erythropoietin production (Celik et al., 2008). The results showed a 1.5-fold improvement in cell concentration with the use of glycerin originating from canola oil when compared to glycerol P.A. Nevertheless, the authors were not able to utilize glycerin derived from soybean due to sample precipitation. Also, the recombinant yeast constructed had the heterologous gene under the control of the inducible pAOX1 promoter, which implies a two-stage process: first to obtain high cell density then to induce heterologous gene production by methanol addition. This was not the case in this study in which a single step was carried out for both biomass and heterologous protein production.

In a second study, constitutive production of a phytase encoding gene cloned under the control of the constitutive pGAP promoter was compared using glucose and glycerin as carbon sources (Tang et al., 2009). In this case, *P. pastoris* had a 30% increase in biomass formation and a 10% increase in recombinant protein production, but the authors reported that the glycerin was pre-treated and this resulted in an effective removal of approximately 85% of the sodium sulfate particles from the original samples (Tang et al., 2009). Therefore, the present study is the first to report the utilization of crude glycerol without any pre-treatment for efficient recombinant protein production.

4. Conclusion

We have shown here for the first time that crude glycerol derived from soybean oil can be utilized for *P. pastoris* growth and constitutive heterologous expression. Yeast grown in crude glycerin samples prepared with sodium hydroxide resulted in 1.5 higher OD than yeast grown in glycerol P.A., although the glycerol utilization rate was about two times slower. Impurity analyses showed that yeast consumes residual methylesters, fatty acids and monoacylglycerols and it uses these compounds as carbon source for growth. Batch fermentations resulted in recombinant α-amylase production concomitant to biomass formation. Together, these results highlight an important application for crude glycerin derived from soybean biodiesel production.

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