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Low cost medium for recombinant endoglucanase II production by *Pichia pastoris*

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The suitability of crude glycerol (75% (v/v) glycerol content) from a biodiesel production plant, and soybean meal and rice bran hydrolysates as replacements in the culture medium for commercially available glycerol, peptone and yeast extract, respectively, to form a low cost culture medium for the growth of, and recombinant endoglucanase II production by, *Pichia pastoris* was evaluated. The use of 1% (v/v) crude glycerol as a carbon source, and a mixture of 0.175% (v/v) soybean meal and 0.15% (v/v) rice bran hydrolysate in the presence of 0.5% (w/v) ammonium sulfate was found to be a suitable medium in the presence of 3% (v/v) methanol as the inducer. Under these conditions, the highest level of extracellular endoglucanase II activity (61.5 U/mL) was found after 120 h culture at 30°C and pH 6.0.

Key words: Biodiesel glycerol, soybean meal, rice bran, endoglucanase II, *Pichia pastoris*.

INTRODUCTION

The high cost of nutrient medium for the cultivation of recombinant *Pichia pastoris* in the production of recombinant proteins is a major factor that needs to be considered and optimized for large scale production (Yadav et al., 2011). Therefore, the development of a suitable culture medium using low cost raw materials is of

interest. The growth and recombinant protein production level in recombinant *P. pastoris* depends on various conditions and on the composition of the culture medium (Batista et al., 2013).

P. pastoris is a methylotrophic yeast that is widely used as a host for the production of recombinant proteins.

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Recently, the improvement of individual cellulase enzymes from *Trichoderma reesei* has been attempted by genetic engineering in *P. pastoris* in order to enhance their cellulose degradation ability (Boonvitthya et al., 2013).

Cellulases, which degrade cellulose, consist of endoglucanases (EGs), cellobiohydrolases and beta-glucosidases. The EGs act on the amorphous regions of cellulose, and EGII is one of the most abundant EGs produced by *T. reesei* and has the highest catalytic efficiency (Qin et al., 2008; Zhang et al., 2012).

The estimated cost of enzyme production at an industrial scale depends to a large extent upon the growth medium used in the process (Hajii et al., 2008; Batista et al., 2013). Previous studies have reported on the fermentation using pure glycerol as the carbon substrate because the salts in raw glycerol exert important inhibitory effects on many microorganisms (Petitdemange et al., 1995). However, yeast strains that can grow on raw glycerol have been reported (Papanikolaou et al., 2002). In order to reduce the medium costs, the crude glycerol produced during biodiesel manufacturing, which contains macro elements, such as calcium, potassium, magnesium, sulfur and sodium (Kitcha and Cheirsilp, 2011), has been used as a carbon source in *P. pastoris* culture medium to increase the cell concentration in the logarithmic growth phase (Celik et al., 2008). In addition, the waste agricultural products of soybean meal and rice bran have been used as organic nitrogen sources since they provide a rich but inexpensive source of nutrients (Xiao et al., 2007; Sereewatthanawut et al., 2008).

The aim of this study was to determine the suitability of crude glycerol, obtained as the by-product of biodiesel production, and soybean meal and rice bran as a substitute for high purity commercial glycerol, peptone and yeast extract, respectively, in the formation of a low cost culture medium for the growth of, and recombinant (r)EGII production by, *P. pastoris*.

MATERIALS AND METHODS

Microbial strain

The genetically engineered *P. pastoris* X-33 yeast strain, which is transfected with the pPICZαA plasmid encoding for the *T. reesei* EGII gene under the methanol inducible AOX1 promoter (Boonvitthya et al., 2013) was provided by the Biocatalysts Research Unit, Chulalongkorn University. The transformed line was maintained on YPD (2% (w/v) peptone, 1% (w/v) yeast extract and 2% (w/v) dextrose) agar. Expression of the rEGII as a secretory product was controlled under the methanol inducible AOX1 promoter and repressed by glycerol.

Preparation of crude glycerol, the byproduct of biodiesel production

The crude glycerol was obtained from the Energy Absolute Public Co. Ltd., biodiesel production plant at Kabinburi Industrial Zone in

Prachinburi, Thailand, which uses refined bleached deodorized palm oil and methanol in the transesterification process with an alkaline catalyst. This crude glycerol preparation contained 75% (v/v) glycerol and so the volume of this crude glycerol added to the medium was set accordingly to give the desired final glycerol concentration (v/v) ignoring the impurities. The crude glycerol was adjusted to pH 7.0 prior to addition to the culture medium.

Preparation of soybean meal and rice bran hydrolysates

Soybean meal was obtained from Lactasoy Co. Ltd., Prachinburi, Thailand, while rice bran was obtained from a rice mill in Pathumthani, Thailand, and separately treated in 1 N sulfuric acid (H₂SO₄) at 33% (w/v). Each mixture was autoclaved at 121°C for 40 min, cooled down and distilled water added to the required volume as well as 10 N NaOH to set the pH to 7.0. The suspension was then filtered and the filtrate stored at 4°C until use. The total nitrogen content was analyzed by the Kjeldahl method (AOAC, 1990).

Medium optimization by a sequential univariate approach

The effect of the nutrient composition, in terms of the concentration of crude glycerol, methanol, soybean meal and rice bran hydrolysates and ammonium sulfate, as well as the medium pH and culture temperature and time, was investigated upon the growth of, and the production of rEGII by, *P. pastoris* in that order as follows.

For the initial growth of *P. pastoris* to form the starter inoculum, the crude glycerol concentration was varied in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer pH 6.0, 0.4% (v/v) *Pichia* trace minerals (PTM) and 1% (v/v) glycerol) by substitution of the pure glycerol with the crude glycerol preparation at a final glycerol concentration of 0, 0.5, 1, 1.5, 2 and 2.5% (v/v). *P. pastoris* was grown in each of these media (50 mL) in 250-mL baffled flasks at 30°C with shaking at 250 rpm for 21 h. The growth of the yeast was then monitored in terms of the yeast dry cell weight (DCW) and optical density, as detailed below.

For the induction of rEGII production by the recombinant *P. pastoris*, the yeast was first grown in the optimum crude glycerol concentration supplemented BMGY medium (BMcGY), harvested by centrifugation (10,000 xg, 3 min), washed in sterile water and then resuspended and transferred into 100 mL BMMY medium (as per BMGY except the glycerol was replaced with methanol) except that the methanol concentration was varied at 0, 0.5, 1, 2, 3 and 4% (v/v) for induction of the AOX-1 promoter and as a carbon source for the yeast. The cell suspension was then grown in 250-mL baffled flasks at 30°C with shaking at 250 rpm for 144 h. For each selected methanol concentration, the same amount (final concentration) of fresh methanol was added to the culture each day (0, 24, 48, 72, 96 and 120 h). At 12, 24 h and then every 24 h thereafter a 5-mL aliquot of the cell suspension was removed to ascertain the yeast DCW and extracellular rEGII activity.

The effect of substitution of peptone with soybean meal hydrolysate (at 0, 0.025, 0.05, 0.1, 0.125, 0.15, 0.175 and 0.2 (v/v)) upon *P. pastoris* growth in the BMcGY medium (optimal crude glycerol level determined as above), and upon the subsequent growth and rEGII production level in BMMY medium was then evaluated as above, except using the optimal methanol concentration in the BMMY medium, respectively.

The replacement of both the peptone and yeast extract in the BMGY and BMMY media with a combined organic nitrogen source of soybean meal and rice bran hydrolysates was then evaluated as above, except using the optimal soybean hydrolysate level and varying the rice bran hydrolysate at 0, 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0% (v/v) in place of the yeast extract.

Table 1. Effect of the crude glycerol concentration as a replacement for pure glycerol in BMGY medium on the growth of recombinant *P. pastoris*.

Crude glycerol concentration (% (v/v))	Dry cell weight (g/L) ^a
0	2.08 ± 0.07
0.5	4.02 ± 0.10
1.0	8.17 ± 0.06
1.5	7.61 ± 0.13
2.0	6.22 ± 0.03
2.5	5.58 ± 0.17

^aData are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

Next, the optimal modified BMcGY and BMMY media were supplemented with ammonium sulfate at 0, 0.1, 0.5, 1, 1.5 and 2% (w/v) and the growth or growth and rEGII production levels, respectively, were evaluated as above. Finally, the effect of the pH (5.5 to 7.0) of the optimal media, followed by that of the cultivation temperature (25 to 35°C), upon the recombinant *P. pastoris* growth and rEGII production levels was evaluated in the same manner.

Analytical methods

Enzyme assay

The evaluation of the extracellular rEGII activity in the culture supernatant assay was performed by monitoring the hydrolysis of carboxymethyl cellulose (CMC) as the total reducing sugar, evaluated using the dinitrosalicylic acid DNS method (Miller, 1959). Each reaction contained 0.5 mL of 2% (w/v) CMC solution in 50 mM acetate buffer (pH 4.8) and 0.5 mL of the diluted test enzyme solution. The reaction was incubated at 50°C for 30 min and then 3 mL of DNS reagent was added, incubated in a boiling water bath for 5 min, and then cooled. Thereafter, the absorbance was measured at 540 nm to estimate the quantity of reducing sugars produced in the assay (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar/mL/min under the above assay conditions. Glucose (0-2 μ g/mL) was used to form the standard curve.

Measurement of protein concentration

The total protein content was determined following the method of Bradford (1976) using bovine serum albumin (0-25 μ g/mL) as a standard and measuring the absorbance at 595 nm with a microplate reader (ANTHOS Zenyth 200, USA).

Determination of yeast cell concentration

The yeast DCW was determined by centrifugation (5000 xg for 5 min) of 5 mL of the culture broth, washing the cells with distilled water, and then drying at 105°C for 24 h. From the obtained mass and the starting culture volume analyzed the DCW was then calculated (g/L). In addition, the yeast cell concentration was estimated in terms of the optical density (OD) of the suspension at 600 nm (OD₆₀₀) AS compared to the medium alone (Charoenrat et al., 2013).

Data analysis

Data WERE presented as the mean ± one standard deviation (SD). The statistical significance of the difference between means was tested by analysis of variance (ANOVA) and Duncan's multiple range tests (DMRT), with significance accepted at the $P < 0.05$ level.

RESULTS AND DISCUSSION

In this study, different cultivation media compositions and cultivation conditions were evaluated in order to attempt to reduce the cost of efficient rEGII production by *P. pastoris*. The culture medium composition is known to be a major influence on the growth and enzyme production, especially the carbon and nitrogen sources (Broach, 2012; Charoenrat et al., 2013).

Effectiveness of crude glycerol to replace commercially available glycerol in high cell density cultivation of *P. pastoris* in BMGY medium

Glycerol is regularly used as the principal initial carbon source in *P. pastoris* cultivation in order to increase the cell concentration. When utilizing the cheaper crude glycerol, a surplus byproduct of the expanding biodiesel production industry, in the BMGY medium, the highest level of yeast biomass production (8.17 g/L, DCW basis) was obtained with 1% (v/v) of biodiesel glycerol (Table 1). Crude glycerol concentrations above or below 1% (v/v) resulted in a lower yeast yield, in accord with the previously reported repression of yeast growth at high crude glycerol levels (Tang et al., 2009). Accordingly, *P. pastoris* was initially grown in BMGY medium with 1% (v/v) crude glycerol in place of the commercial glycerol (hereafter called BMcGY). Although this was a similar glycerol concentration to the commercial glycerol containing BMcGY medium, a likely economic saving would still be gained since, as compared to the pure glycerol (US\$1.11 per kg), the crude glycerol is some ten-fold cheaper (US\$0.11 per kg). Indeed, as a cheaper carbon source for biotechnological applications that may make them economically feasible, crude glycerol has been used as carbon source in many industrial fermentations (Chatzifragkou et al., 2011). However, the crude glycerol derived from biodiesel production typically possesses a low glycerol concentration of low value because of the impurities. Further refining of the crude glycerol will depend on the economy of production scale and/or the availability of a glycerol purification facility (Naresh and Brian, 2006).

Effects of the methanol concentration on BMMY medium

Methanol is typically the widely accepted industrial choice

Figure 1. Effect of the methanol concentration on the growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* in BMMY medium. Data are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments.

Table 2. The growth of recombinant *P. pastoris* in BMcGY^a medium with different soybean meal hydrolysate concentrations as a replacement for the peptone.

Soybean meal hydrolysate (% (v/v))	Dry cell weight (g/L) ^b
0.0000	4.57 $\pm$ 0.17
0.0125	5.35 $\pm$ 0.05
0.0250	6.21 $\pm$ 0.10
0.0500	6.63 $\pm$ 0.13
0.0750	7.04 $\pm$ 0.09
0.1000	7.25 $\pm$ 0.10
0.1250	7.41 $\pm$ 0.11
0.1500	7.67 $\pm$ 0.04
0.1750	8.01 $\pm$ 0.13
0.2000	6.13 $\pm$ 0.12

^aBMcGY = BMGY medium but with the 2% (v/v) pure glycerol replaced with 1% (v/v) crude glycerol (75% (v/v) glycerol) from a biodiesel production plant. ^bData are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

of carbon source for *P. pastoris* fermentations, and also serves as the inducer for recombinant protein expression under the control of the AOX1 promoter of *P. pastoris*. Following the initial growth of *P. pastoris* in BMcGY medium and transfer to BMMY medium without methanol no rEGII production was detected, as expected with the

AOX1 promoter. Increasing the methanol concentration up to 3% (v/v) correspondingly increased the rEGII level produced, especially at 2 and 3% (v/v) methanol, with the highest rEGII level obtained (68.5 U/mL) at 3% (v/v) methanol after 120 h (Figure 1). Increasing the methanol concentration further to 4% (v/v), however, resulted in almost no rEGII production, consistent with that previously reported in the production of human serum albumen in this yeast (Bushell et al., 2003). Accordingly, a methanol concentration of 3% (v/v) was used in the BMMY medium (hereafter called BMM₃Y).

Effect of organic nitrogen sources

As determined by Kjeldahl's method, the total nitrogen content of the soybean meal and rice bran hydrolysates were 0.52 and 0.31% (w/v), respectively, as compared to ~12% and ~9.5% (w/v) (Technical Data from HiMedia Laboratories Pvt. Ltd.) for the peptone and yeast extract, respectively.

Soybean meal hydrolysate replacement for peptone

The use of soybean meal hydrolysate in place of peptone in the BMcGY medium was found to support the growth of *P. pastoris*, with an increasing yeast yield being obtained with an increasing soybean meal hydrolysate concentration up to a maximum yeast yield (8.01 g/L, DCW basis) at 0.175% (v/v) soybean hydrolysate (Table 2). This compared reasonably well with the 8.17 g/L (DCW basis) obtained with the peptone containing

Figure 2. The growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* over time when grown in BMM₃YS media (soybean meal hydrolysate at 0.175% (v/v) as a replacement for peptone in the BMM₃Y media). Data are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments.

Table 3. Effect of increasing rice bran hydrolysate concentration as a replacement for yeast extract in BMcGYS^a medium on the growth of recombinant *P. pastoris*.

Rice bran hydrolysate (% (v/v))	Dry cell weight (g/L) ^b
0.0000	4.86 $\pm$ 0.12
0.0125	5.28 $\pm$ 0.15
0.0250	6.12 $\pm$ 0.19
0.0500	6.48 $\pm$ 0.05
0.0750	6.93 $\pm$ 0.09
0.1000	7.14 $\pm$ 0.23
0.1250	7.32 $\pm$ 0.09
0.1500	8.12 $\pm$ 0.11
0.1750	7.81 $\pm$ 0.22
0.2000	7.46 $\pm$ 0.10

^a BMcGYS = BMGY medium but with the 2% (v/v) pure glycerol and 1% (w/v) peptone replaced with 1% (v/v) crude glycerol (75% (v/v) glycerol) from a biodiesel production plant and 0.175% (v/v) soy meal hydrolysate, respectively.

^b Data are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

BMcGY medium. Accordingly, the BMcGY and BMM₃Y media were modified to contain 0.175% (v/v) soybean meal hydrolysate in place of peptone (hereafter called BMcGYS and BMM₃YS, respectively).

When *P. pastoris* was cultured and induced in the BMM₃YS medium, the modified medium was found to support rEGII production, with a maximum level (59.7 U/mL) being obtained after 120 h (Figure 2). Although this was 1.15-fold lower than that with the peptone containing BMM₃Y medium, a likely economic saving is still gained (~99.4% decrease; [http: //www.quotenet.com/commodities/soybean-meal-price](http://www.quotenet.com/commodities/soybean-meal-price) and HiMedia Laboratories Pvt. Ltd.). Note that the yeast yield obtained in the modified BMM₃YS medium essentially correlated with the obtained rEGII production level. Thus, soybean meal hydrolysate has the potential to be employed as a peptone replacement.

Rice bran hydrolysate replacement for yeast extract

The use of rice bran hydrolysate in place of peptone in the modified BMcGYS medium was found to support the growth of *P. pastoris*, with an increasing yeast yield being obtained with increasing soybean meal hydrolysate concentrations up to a maximum (8.12 g/L, DCW basis) at 0.15% (v/v) (Table 3). This compared reasonably well with the 8.17 g/L (DCW basis) obtained with the peptone containing BMcGY medium. Accordingly, the BMcGYS and BMM₃YS media were further modified to contain 0.15% (w/v) rice bran hydrolysate in place of yeast extract, hereafter called BMcGYSR and BMM₃YSR, respectively.

When *P. pastoris* was cultured and induced in the BMM₃YSR medium, the modified medium was found to support rEGII production reasonably well but not to as

Figure 3. The growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* over time when grown in BMM₃YSR medium (rice bran hydrolysate at 0.15% (w/v) as a replacement for yeast extract in BMM₃YS media). Data are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments.

Table 4. Effect of ammonium sulfate as an inorganic nitrogen supplement in the BMcGYTRS^a medium on the growth of recombinant *P. pastoris*.

Ammonium sulfate (% (w/v))	Dry cell weight (g/L) ^b
0.00	4.52 $\pm$ 0.07
0.10	8.26 $\pm$ 0.47
0.25	8.30 $\pm$ 0.12
0.50	8.34 $\pm$ 0.18
1.00	7.81 $\pm$ 0.26
1.50	7.66 $\pm$ 0.09
2.00	7.76 $\pm$ 0.13

^aBMcGYRS = BMGY medium but with the 2% (v/v) pure glycerol, 1% (w/v) peptone and 2% (w/v) yeast extract replaced with 1% (v/v) crude glycerol (75% (v/v) glycerol) from a biodiesel production plant, 0.175% (v/v) soy meal hydrolysate and 0.15% (v/v) rice bran hydrolysate, respectively. ^bData are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

in the BMM₃YS and BMM₃Y media, respectively, this loss of yield was still not as great as the likely financial savings gained (~98.4%; <http://www.alibaba.com/Rice-Bran> and HiMedia Laboratories Pvt. Ltd.). Therefore, the mixture of soybean meal and rice bran hydrolysates is a cheap nitrogen source that could be used for enzyme production.

Effect of the inorganic nitrogen source

The effect of supplementing the BMcGYSR medium with ammonium sulphate, as an inorganic nitrogen source, on the growth of *P. pastoris* was evaluated over the range of 0-2% (w/v). Increasing the ammonium sulphate concentration up to 0.5% (w/v) caused an increased yield of yeast, reaching a maximum of 8.34 g/L (DCW basis) (Table 4). Further increasing the ammonium sulphate concentration above 0.5% (w/v) reduced the yeast yield and so 0.5% (w/v) was taken as optimal for both media (hereafter called BMcGYSRa and BMM₃YSRa).

When the recombinant *P. pastoris* was cultured and induced in the BMM₃YSRa medium an increased yeast yield and rEGII production level, ~1.1-fold to 6.53 g/L and 61.7 U/mL, respectively was observed as compared to that in the BMM₃YSR medium without ammonium sulphate (Figure 4). This result is in agreement with previous reports that too high or too low ammonium ion concentration in the culture medium leads to a reduced cell growth (Cos et al., 2006; Yalcin and Ozbas, 2008).

high an extent as in the other media. A maximum rEGII level (54.0 U/mL) was obtained after 120 h (Figure 3). Although the maximum level of obtained rEGII was 1.11- and 1.27-fold lower than the maximum amount obtained

Figure 4. The growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* over time when grown in BMM₃YSRA media (BMM₃YSR with 0.5 (w/v) ammonium sulphate). Data are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments.

Table 5. Effect of the medium pH on the growth of, and recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* in BMM₃ YRSA^a medium.

pH	Dry cell weight (g/L) ^b	Endoglucanase II activity (U/mL) ^b
5.5	6.57 $\pm$ 0.18	60.30 $\pm$ 0.33
6.0	6.61 $\pm$ 0.04	61.42 $\pm$ 2.28
6.5	6.73 $\pm$ 0.22	24.07 $\pm$ 2.78
7.0	6.69 $\pm$ 0.22	19.78 $\pm$ 2.07

^aBMM₃YSRA = BMMY medium but with the 0.5% (v/v) methanol, 1% (w/v) peptone and 2% (w/v) yeast extract and replaced with 3% (v/v) methanol, 0.175% (v/v) soy meal hydrolysate and 0.15% (v/v) rice bran hydrolysate, respectively, and supplemented with 0.5% (w/v) ammonium sulphate; ^bData are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

Effect of the culture medium pH

With respect to the medium pH, no significant effect on the recombinant *P. pastoris* yield in the BMM₃YSRA medium was observed at pH values between 5.5 and 7.0 (Table 5). This is consistent with the reported ability of *P. pastoris* to grow within the pH range of 3.0-7.0 (Inan et al., 1999; Bayraktar, 2009). However, the level of obtained extracellular rEGII activity was markedly reduced at pH 6.5 (~2.5-fold) and pH 7.0 (~3.1-fold) as compared to that at pH 5.5-6.0 (Table 5), which may

Table 6. Effect of the incubation temperature on the growth of, and recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* in BMM₃YSRA^a medium.

Temperature (°C)	Dry cell weight (g/L) ^b	Endoglucanase II activity (U/mL) ^b
25	6.21 $\pm$ 0.12	58.95 $\pm$ 1.45
30	6.67 $\pm$ 0.26	61.49 $\pm$ 0.25
35	1.17 $\pm$ 0.09	7.87 $\pm$ 0.14

^aBMM₃YSRA = as per Table 5; ^bData are shown as the mean $\pm$ 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

reflect a higher protease activity in the culture medium or a decreased enzyme stability at the higher pH values (Cos et al., 2006).

Effect of incubation temperature

With respect to the cultivation temperature, the optimal yeast growth (as DCW) and rEGII production level was obtained at 30°C for the culturing and induction of the recombinant *P. pastoris* in the BMM₃YSRA medium (Table 6). Increasing the culture temperature from 30 to 35°C resulted in a marked (5.7- and 7.8-fold) decrease in the yeast and rEGII yields obtained, respectively. In agreement, it has previously been reported that 30°C was the most appropriate template for the growth of, and enzyme production by, *P. pastoris* in general (Shi et al., 2003; Macauley et al., 2005; Rahbarizadeh et al., 2006).

Conclusions

The culturing of recombinant *P. pastoris* for the production of rEGII was attained using BMcGYRS and BMM₃YRS media, which are the use of a 1% (v/v) crude glycerol (75% (v/v) glycerol content from a biodiesel production plant) plus 0.175% (v/v) soybean meal and 0.15% (v/v) rice bran hydrolysates as a replacement for the 2% (v/v) glycerol, 2% (w/v) peptone and 1% (w/v) yeast extract in standard BMGY and BMMY media, respectively. Although the optimal rEGII production required supplementation of this modified medium with 0.5% (w/v) ammonium sulphate, overall these media will provide a likely economic advantage for the industrial scale production of recombinant enzymes by *P. pastoris*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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