Cloning and Expression of a Functional Core Streptavidin in *Pichia pastoris:* Strategies to Increase Yield

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Streptavidin is widely used as an analytical tool and affinity tag together with biotinylated surfaces or molecules. We report for the first time a simple strategy that yields high biomass of a Pichia pastoris *strain containing a methanol induced core streptavidin (cStp) gene. Three factors were evaluated for biomass production: glycerol concentration, aeration, and feed flow rates in a bioreactor. Recycling of recombinant cells, either free or immobilized, was investigated during induction. Concentration of 2.0 M glycerol, feeding flow rate of 0.11 mL min^{-1}, and aeration by air injection dispersed with a porous stone combined with agitation at 500 rpm were the set of conditions resulting into maximum biomass yield (150 g L^{-1}). These parameters yielded 4.0 g L^{-1} of cStp, after 96 h of induction. Recombinant biomass was recycled twice before being discarded, which can reduce production costs and simplify the process. Immobilized* P. pastoris *biomass produced 2.94 and 1.70 g L^{-1} of cStp in the first and second induction cycle, respectively. Immobilization and recycling of recombinant* P. pastoris *biomass opens new possibilities as a potential strategy to improve volumetric productivity for heterologous protein expression.*
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Keywords: Pichia pastoris*, streptadivin, heterologous protein expression, recycling of biomass, aeration*

Introduction

Streptavidin is a 60-kDa tetrameric protein that can bind up to four biotin molecules and has one of the highest and most stable noncovalent affinity constants.[1] This property has attracted interest in the construction of chimeric proteins fused with the streptavidin affinity domain to allow simultaneous isolation and immobilization of proteins.[2–5] Applications include immunoassays, hybridization assays, immunotherapy, localization of antigens, bioremediation, and affinity chromatography.[6–8] Some enzymes such as β-galactosidase[9] and lipase[10] fused to streptavidin have been reported to be produced in *Escherichia coli* and simultaneously isolated and immobilized on biotinylated silica. However, if the interest is to fuse the biotin affinity domain of streptavidin to more complex eukaryotic proteins, the lack of an endomembrane system in bacteria does not assure correct processing of the protein.[11–13]

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Pichia pastoris expression system continues to be one of the most successfully used systems for high level expression of heterologous proteins. It is based on the alcohol oxidase promoter (P_{AOX1}), which is induced by methanol and repressed by carbon sources such as glucose and glycerol.[12,14] This repression of P_{AOX1} is important to produce maximum biomass prior to induction and accumulation of heterologous proteins. Several strategies have been proposed to increase cell mass concentration and consequently heterologous protein yield in *P. pastoris*.[15,16] It has been suggested that cell density reaches its maximum during the glycerol batch phase due to the depletion of salts in the growth medium, which impairs glycerol utilization.[17] However, another study has shown an increase in biomass from the batch phase, in which glycerol is completely exhausted, to the end of the induction phase, where a mix of trace minerals and methanol is fed as the only carbon and energy source.[18] This study also highlights that the batch fermentation medium formulation had a reduced basal salts concentration compared to the common *P. pastoris* media recipes. Together, these studies indicate a need

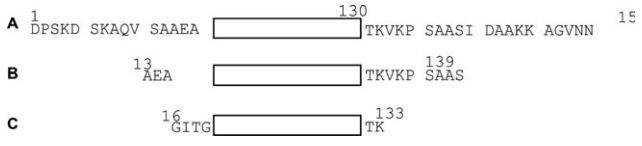

Figure 1. Schematic representation of the different forms of Streptavidin.

Open box indicates residues Thr-21 through Phe-130. A. Amino acid sequence of a single streptavidin subunit (159 residues).[8] B. Naturally occurring core Streptavidin amino acid sequence (127 residues).[23] C. Core Streptavidin amino acid sequence used in this study (118 residues).[5]

for careful media formulation regarding batch and induction phase strategies, which are usually recombinant protein dependent. Efficient oxygenation is another important aspect for *P. pastoris* cultures, and has been shown to significantly increase biomass and production of human serum albumin.[19]

One interesting alternative to increase volumetric productivity of recombinant proteins is the immobilization of biomass, which offers a means of recycling the cells, thereby decreasing costs in the production process.[20,21] Calcium alginate encapsulation of cellular biomass is one example of a simple and low cost immobilization strategy that provides controlled solute permeability due to the solid matrix physicochemical characteristics.[21,22] The number of cells per culture unit volume can be increased by calcium alginate immobilization, as well as decoupling of dilution and growth rates in continuous cultures. Alteriis et al.[20] immobilized cells of recombinant *Kluyveromyces lactis* in calcium alginate spheres for glucoamylase production. Cells were shown to be active after 3 months of storage at 4°C without significant loss in metabolic activity and enzyme production remained constant. However, the authors suggest that constant monitoring of aeration is a key variable for optimal results. One potential candidate of biomass immobilization for recombinant streptavidin expression is *P. pastoris*, which has not been studied under this particular condition.

Despite numerous applications of streptavidin, its heterologous production in *P. pastoris* has not been documented. In this study, a *P. pastoris* KM71 strain expressing the biotin binding domain of streptavidin (core streptavidin) was constructed. We used a fermentation medium and a simple procedure to culture the yeast, which includes a fed-batch with air injection and stirring to maximize cell mass yield. Finally, recycling of recombinant cell mass immobilized in calcium alginate beads was evaluated. This particular use of *P. pastoris* cell mass, which can decrease cultivation time and improve induction for recombinant protein expression, is an important issue in biotechnological processes.

Material and Methods

Strains, vectors, and culture medium

For recombinant protein expression, the *P. pastoris* KM71 (*his*$^-$, Mut^s) strain and expression plasmid pPIC9 were used (Invitrogen®) Plasmid pSTP4, containing the full-length streptavidin gene, was used to obtain the sequence that codes for the core streptavidin (cStp).[9]

YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) was used as a general culture medium. For protein expression experiment's, cells were grown in BMG medium [13.4 g L^{-1} YNB medium (Sigma®, St. Louis, USA), 100 mM potassium phosphate, (pH 5.0); 4.0 × 10^{-5}% (v/v) biotin and 0.27 M glycerol]. The culture was fed with sterilized basal salts media [23 g L^{-1} $CaSO_4$-$2H_2O_4$, 55 g L^{-1} K_2SO_4, 3.73 g L^{-1} $MgSO_4$-$7H_2O$, 1.03 g L^{-1} KOH, 6.68 mL L^{-1} H_3PO_4, 5.0 g L^{-1} glycerol, and 0.5 mL L^{-1} Antifoam 204 (Sigma®, St. Louis, USA)] supplemented with PTM_1 (24 mM $CuSO_4$, 0.53 mM NaI, 19.87 mM $MnSO_4$, 0.83 mM Na_2MoO_4, 0.32 mM boric acid, 2.1 mM $CaCl_2$, 0.15 mM $ZnCl_2$, 0.23 M $FeSO_4$, and 0.82 mM biotin) at 4.35 mL L^{-1}.

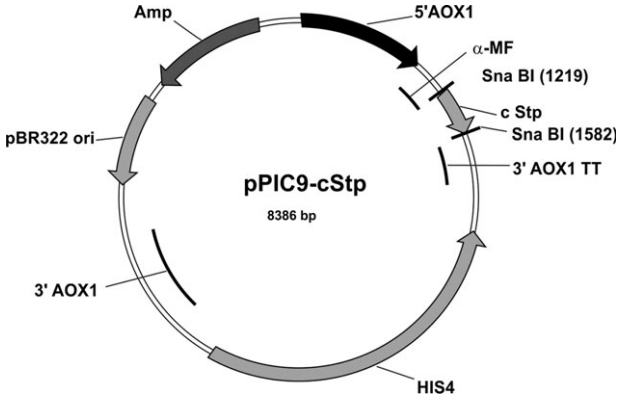

Figure 2. Map of the expression vector pPIC9-cStp.

The *cStp* coding sequence was cloned in frame with the α-mating factor secretion signal (a-MF) in the *Sna*BI restriction site (see Materials and Methods for details). The 5′*AOX1* = *AOX1* promoter; 3′*AOX1*TT = transcription termination site from *AOX1*; *HIS4* = histidinol dehydrogenase gene; 3′*AOX1* = 3′ region of *AOX1*; Amp = Ampicillin resistance gene; pBR322 = *E. coli* origin of replication.

After reaching a wet cell weight of 150 g L^{-1}, cells were harvested at 4,500*g* for 4 min at 4°C and resuspended in BMM medium [13.4 g L^{-1} YNB, 1.0 M sodium citrate buffer (pH 3.0), 4.0 × 10^{-5}% (v/v) biotin and 0.5% (v/v) methanol] for induction. Methanol was added to the culture every 24 h at a final concentration of 0.5% (v/v). The induction phase was carried out for 144 h.

Cloning and transformation of P. pastoris *with the core streptavidin gene*

The DNA fragment coding for 118 residues (Figures 1C) of cStp, according to Sano et al.,[5] was amplified by PCR with the primers: CORESTEP-F 5′-TAC GTA GGC ATC ACC GGC ACC TGG TAC AAC CAG-3′ and CORESTEP-R 5′-TAC GTA **CTA** CAC CTT GGT GAA GGT GTC GTG-3′. The reverse primer creates a stop codon (bold in CORESTEP-R). Both primers create a restriction site for *SnaBI* (underlined), which allows for insertion at the *SnaBI* site of pPIC9. The cStp coding sequence was cloned into the *SnaBI* site of pPIC9, resulting in pPIC9-cStp (Figure 2). Verification of the correct *cStp* orientation was done by restriction analysis with *SnaBI* and *BglII*. pPIC9-cStp was then linearized with *SalI* and used to transform *P. pastoris* KM71 by electroporation, according to the Invitrogen® *P. pastoris* manual. Positive clones were selected in minimal media devoid of histidine and confirmed by PCR. Negative controls were constructed by transforming *P. pastoris* KM71 with SalI-linearized pPIC9.

Initial glycerol concentration and feeding flow rates on biomass production

Single colonies of *P. pastoris* KM71/pPIC9-cStp and *P. pastoris* KM71/pPIC9, grown on YPD plates, were used to

inoculate 50 mL BMG medium in a 250 mL Erlenmeyer flask. This preculture was incubated overnight at 25°C and 250 rpm on a rotary shaker. To define kinetic parameters of specific growth rate and saturation constants the preculture was used to initiate 50 mL cultures in 250 mL Erlenmeyer flasks with an initial OD_{600} of 0.2, containing BMG medium with glycerol concentrations of 0.03, 0.05, 0.11, 0.13, 0.27, and 0.40 M. In addition, glycerol concentrations of 0.67, 1.07, 1.34, 1.62, 2.0, and 2.67 M in BMG were used to investigate glycerol inhibition of yeast growth, thereby defining the fed-batch bioreactor operation conditions. The concentration of glycerol resulting in maximum biomass within 24 h of culture was used to construct the time course growth curve, which allowed calculation of the feeding flow rate, by considering the specific growth rate equal to the dilution rate from the equation $D = F/V$ (where D is the dilution rate in 1/h, F is the flow rate in L/h and V the reactor volume in liters).

Initially *P. pastoris* KM71/cStp was pre-cultured in 50 mL BMG medium with 0.67 M glycerol in a 250 mL shake flask. The culture was incubated at 250 rpm and 25°C for 12 h. This 50-mL culture was then used to inoculate a 1.0 L Wheaton spinner flask (Wheaton® Biostir) containing 200 mL of BMG medium. The feed rate used was selected among the combination of three feeding rates (0.11, 0.22, and 0.5 mL min^{-1}) and four final glycerol concentrations (1.07, 1.34, 2.0, and 2.67 M). The two glycerol concentrations and the two feed rates resulting in maximum biomass yield were selected for testing with two types of aeration: air injection and stirring.

Effect of aeration on biomass production

P. pastoris KM71/cStp and the negative control were grown in 50 mL of BMG medium containing 0.67 M glycerol, as previously described. This preculture was then used to inoculate 150 mL of BMG medium in a 1.0 L Wheaton spinner flask placed over stir plates. Aeration of the spinner flasks was done by injecting air at the 4 VVM, through silicone tubing adhered to the internal reactor wall and a porous stone. Two mixing velocities were tested: 250 and 500 rpm. The culture was fed at either 0.11 or 0.22 mL min^{-1}, with the basal salts medium containing glycerol at concentrations of 1.34 and 2.0 M until it reached a final volume of 400 mL.

Biomass yield, immobilization and, production of cStp

Cells were cultured in 1.0 L Wheaton® Biostir containing 200 mL BMG medium with 0.67 M glycerol at an initial OD_{600} of 0.2. The culture was fed with fresh media (basal salts and PTM_1) at a flow rate of 0.11 or 0.22 mL min^{-1}, until it reached a final volume of 400 mL in the bioreactor. Feeding was then interrupted and the biomass harvested at 4,500*g*, 4°C, for 10 min and resuspended in 200 mL of 0.01% peptone water. A 100 mL volume of this suspension was immobilized in calcium alginate beads as previously described.[22] Both the immobilized and remaining peptone water cell suspension fractions were then subjected to the induction process, in parallel, in bioreactors containing 400 mL modified BMM medium [13.4% g L^{-1} YNB; 1.0 M sodium citrate (pH 3.0); 4.0×10^{-5}% (v/v) biotin and methanol to a final concentration of 0.5% (v/v)] at 30°C for 144 h. Supernatant samples of the induced cultures were collected every 24 h and kept at −80°C until analysis. Methanol was added at a final concentration of 0.5% (v/v) every 24 h throughout the process to maintain induction. Total secreted protein from culture samples was analyzed by Bradford,[24] SDS-PAGE,[8] and a biotin binding assay.[25] At the end of the first batch of induction, the beads containing the immobilized cells as well as the free suspended cells were washed with phosphate buffer and centrifuged at 4,500*g* and 4°C for 10 min and kept at 4°C submersed in a 2% (v/v) calcium chloride solution for 24 h. These were then reused for a new batch of induction.

Functional analysis of recombinant streptavidin

To detect secreted cStp and analyze its ability to bind biotin, biotinylated silica particles were prepared as previously described.[25] Cleared culture supernatant from induced *P. pastoris* KM71/cStp was first reacted with HABA (2′ hydroxy-amino-benzene-4′carboxilic acid; PIERCE®). The mixture was then placed in contact with the biotinylated silica particles. Biotin displaces cStp from the HABA reagent and changes the color of the suspension. After the reaction has taken place, the mixture is measured at 420 nm and compared to the previously registered absorbance of the complex HABA-streptavidin. In addition to using *P. pastoris* KM71/pPIC9 supernatant as a negative control, distilled water was also used. To determine the concentration of secreted cStp in the supernatant of *P. pastoris* KM71/cStp, a standard avidin curve was constructed.[26] A 200-μL aliquot of the induced culture was mixed with 50 μL of HABA. This reaction mixture was left for 5 min at room temperature and the absorbance at 500 nm (A_1) measured. Nearly 50 μL volume of biotin (500 μM) was then added to the reaction mixture and left for an additional 5 min. A new measurement at 500 nm was taken (A_2). The difference $A_1 - A_2$ (As) was registered and subjected to the standard curve, which was used to obtain the concentration of cStp in the induced culture supernatant as mg mL^{-1}.

Results and Discussion

Effect of glycerol concentration and flow rate during the fed batch biomass production phase

Maximum specific growth rate of the recombinant strain *P. pastoris* KM71/cStp at various glycerol concentrations after 24 h of shake flask culture is presented in Figure 3. The maximum specific growth rate was obtained with 0.67 M glycerol in BMG medium, which infers maximum cell mass yield. Further increase in glycerol concentration affected negatively the specific growth rate, indicating that high glycerol concentration has an inhibitory effect on the growth of the recombinant strain.

Figure 4 shows growth rates of the recombinant strain in BMG with 0.67 M glycerol in shake flasks. These growth rates were used to set the dilution rate of the fed batch culture. This fed-batch culture was then carried out in a bioreactor with a starting OD_{600} of 0.2 and fed with BMG medium, the PTM_1 solution and glycerol at a feed flow rate of 0.11, 0.22, and 0.5 mL min^{-1} to a final glycerol concentration of 1.06, 1.34, 2.0, and 2.67 M, respectively (Table 1). The results indicate that the combination of the flow rates of 0.11 and 0.22 mL min^{-1} and the glycerol concentration of 1.34 and 2.0 M resulted in higher biomass yield. These conditions were selected to evaluate the effect of aeration and agitation speed in the spinner flasks for biomass production.

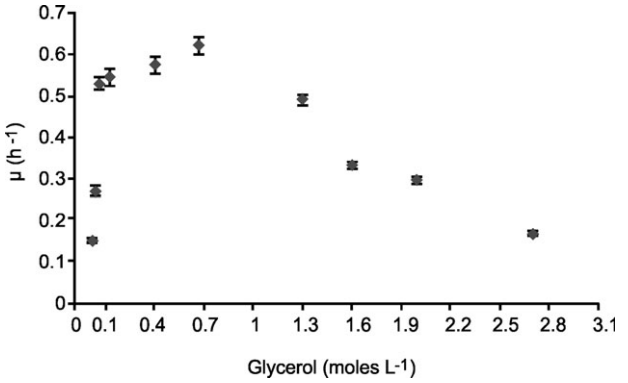

Figure 3. Specific growth rate (μ) of *P. pastoris* KM71/cStp as a function of glycerol concentration in the culture medium.

Each point is the average of the slope of the initial linear regression of triplicates cultures of recombinant *P. pastoris* KM71/cStp culture taken from the plot $\ln OD_{600}$ vs. Time (h). A 24-h culture in BMG medium initiated the cultures at an OD_{600} of ~0.2.

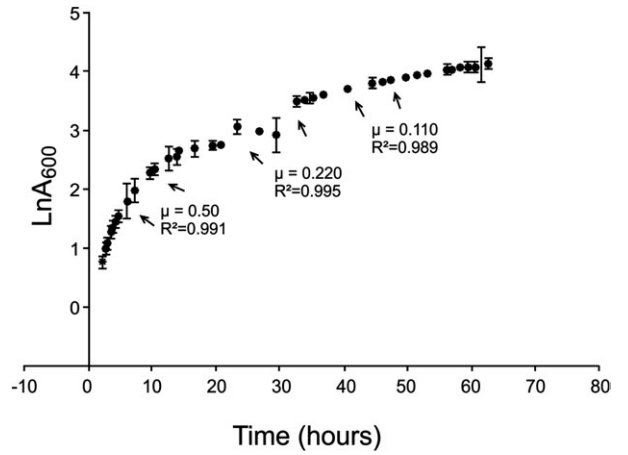

Figure 4. Time course of *P. pastoris* KM71/cStp batch culture in BMG medium with 0.67 M glycerol.

Specific growth rates (μ) are the angular coefficient of the linear regression of the points in each interval indicated by the arrows. These growth rates were used to set the dilution rate of the fed-batch culture.

Effect of aeration and agitation speed on biomass production in fed-batch bioreactor

The two glycerol concentrations of 1.34 and 2.0 M combined with two feeding flow rates were evaluated with agitation of 250 and 500 rpm. The maximum biomass concentration of 150 g L^{-1} was found with a combination of feeding flow rate of 0.11 mL min^{-1}, 2.0 M glycerol and shaking of 500 rpm (Table 2), indicating that this combination has favored the oxygen mass transfer in the stirred flasks and consequently biomass production. Thus, higher glycerol concentrations and increased aeration through shaking seem to be desirable in achieving higher biomass.

This method was more efficient in terms of biomass accumulation when compared to that of ZHANG et al.,[19] where by using microbubble dispersion for culture aeration, biomass accumulation reached 137 g L^{-1}. Despite using a more complex and laborious setup, biomass accumulation was still lower than that presented here, where a simple porous stone was employed. Thus, the use of a simple sparging system to increase aeration seems to be an alternative to improve mass transfer in spinner flasks.

Table 1. Cell Mass Yield of *P. pastoris* KM71/cStp in Fed-Batch Culture

Feeding Rate (mL min^{-1})	Glycerol (mol L^{-1})	Cell Mass (mg mL^{-1})
0.11	1.06	2.95 (± 0.3)
	1.34	15.38 (± 0.2)
	2.00	59.80 (± 0.5)
	2.67	24.41 (± 0.3)
0.22	1.06	18.30 (± 0.2)
	1.34	30.23 (± 0.4)
	2.00	15.03 (± 0.2)
	2.67	12.92 (± 0.3)
0.50	1.06	9.12 (± 0.3)
	1.34	6.16 (± 0.1)
	2.00	11.05 (± 0.4)
	2.67	7.79 (± 0.2)

A 24-h culture with 36.38 g L^{-1} obtained from yeast grown in a bioreactor (Wheaton® Biostir) containing 200 mL BMG medium with 0.67 M glycerol at an initial OD600 of 0.2 was used as the start culture. Basal salt medium with glycerol at the indicated concentrations was fed to a final culture volume of 400 mL. The cell mass was achieved without an aeration device.

Table 2. Maximum Cell Mass Achieved by *P. pastoris* KM71/cStp in Fed-Batch Culture

		Cell mass (g L^{-1}) Feeding Rate	
		0.11 (mL min^{-1})	0.22 (mL min^{-1})
Glycerol (mol L^{-1})	Speed (r.p.m)	33 h	16 h
1.34	250	68.78 (± 0.02)	48.87 (± 0.03)
	500	72.40 (± 0.04)	65.88 (± 0.02)
2.00	250	118.5 (± 0.08)	96.40 (± 0.03)
	500	150.0 (± 0.02)	107.6 (± 0.06)

Feeding of the fed-batch reactor with basal salt medium containing glycerol started with an initial cell mass concentration of 36.38 g L^{-1}. The system was aerated by magnetic stirring and air injection and dispersion through a porous stone device. The fed-batch culture was terminated at the final volume of 400 mL.

Feeding of BMG medium with PTM_1 solution and glycerol proved to be better than the system described by DIGAN et al.[27] Here it was possible to reach high biomass concentration after activation in BMG followed by feeding directly into the bioreactor. Therefore, initial activation of *P. pastoris* cells in YPD and then growth in BMG prior to bioreactor inoculation can be replaced by a single activation step in BMG followed by bioreactor inoculation. In addition, the biomass of 150 g L^{-1} obtained in spinner flasks using this minimal medium, air injection and magnetic stir agitation was comparable to results (170 g L^{-1}) that have been obtained in larger scale batch fermentors with a dissolved oxygen control at 40% oxygen saturation maintained by pure oxygen injection and increased agitation.[18]

Production of cStp by P. pastoris

The biomass obtained from the previously described conditions was used for methanol induction as either free suspended form or as immobilized beads. The extracellular protein profile is shown in Figure 7. In both treatments a 14 kDa band corresponding to cStp was observed, which is not present in the control (*P. pastoris* KM71/pPIC9). The extracellular cStp was detected as early as 24 h after induction. Similar results have been reported for the antibody fragment

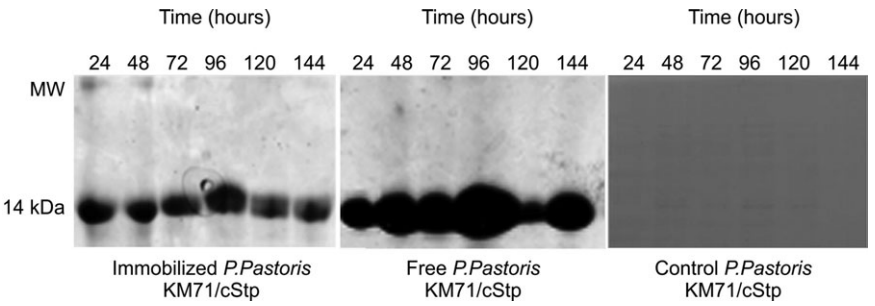

Figure 5. Comassie stained SDS-PAGE of cleared culture supernatant from free and immobilized *P. pastoris* KM71/cStp cells sampled from time periods during the induction phase.

Equal amounts of cell mass produced during fed-batch culture were resuspended into the methanol induction medium as free or calcium alginate-immobilized cells.

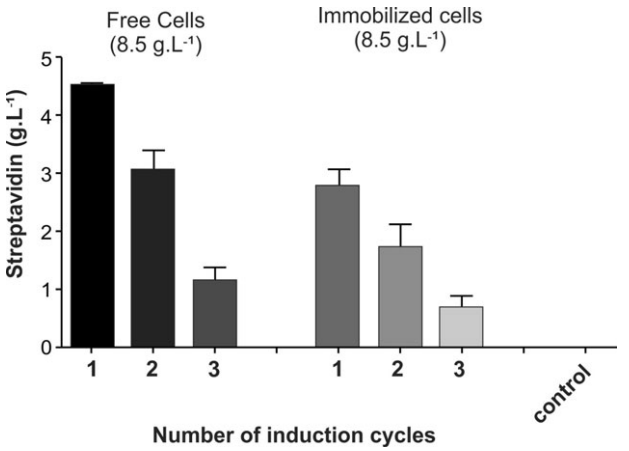

Figure 6. Production of cStp with three cycles of 96 h of induction from free and calcium alginate-immobilized *P. pastoris* KM71/cStp.

A33scFv and a bovine enterokinase in *P. pastoris*.[20,28] A decrease in cStp in culture supernatants after 96 h of induction can be due to the proteolitic activity of native *P. pastoris* proteases and stationary phase of culture.

An increase of 55.9 g L^{-1} in biomass during the induction phase was observed when suspended free cells were used (data not shown). This increase in biomass during the induction phase is not unexpected as methanol is also used for cell growth.[20,28–30] This suggests that adjusting methanol feeding rate could potentially increase protein expression after 96 h.

Suspended free recombinant cells produce more heterologous protein than immobilized recombinant cells (Figures 5 and 6), probably due to limitations on substrate and product diffusion, which is inherent to immobilization by entrapment in the polymerizing gel.[31] Taking into account the cell mass data extracted from Figure 6 it can estimated a productivity achieved by reusing the immobilized cell mass of about 80% of that achieved by reusing the free cell mass. It was also observed that cells were released from the immobilized system at the end of the induction period, as a consequence of cells growing faster in the porosity of the alginate gel closer to the bead surface.

The biomass of *P. pastoris* KM71/cStp cultures either free, suspended, or immobilized was recycled twice, maintaining protein profile with the induction medium. However, it was observed that at 96 h of induction, a 30% drop in protein concentration occurred. Figure 6 shows the concentration of cStp at 96 h of induction in the three batch cycles of biomass recycling. The loss in protein production with reutilization of the biomass is not unexpected and can be explained by the loss in culture viability. Figure 7 shows the protein profile of the induction culture supernatant with reutilization of the biomass. Despite the low production of cStp per biomass unit with reutilization, recycling of biomass is still feasible, allowing for recombinant protein production even after the third utilization. There is also potential for improvement of this system by maintaining a viable static culture. The challenge is to shift energy for recombinant protein expression as opposed to cell growth, and this is a subject for further investigation.

Cleared supernatant samples from the induction culture at 96 h were analyzed for cStp binding to biotinylated silica particles. The results are presented in Table 3. The displacement of streptavidin from the HABA reagent by biotin after addition of biotinylated silica beads was demonstrated by a reduction in absorbance at 500 nm (A_2). The higher difference between the absorbance before and after the addition of biotin showed the higher concentration of cStp in the culture medium. This data indicates that cStp maintains its biotin binding capacity, when compared to purified avidin, and shows that this recombinant protein is secreted as a functional molecule that can be used in other applications.

Higher protein yield was verified from the free yeast cell biomass, harvested and subjected to another cycle of induction. Core streptavidin production was verified up to the second cycle of biomass utilization, which is already a promising indication of the potential of the immobilized system. An improvement of this system certainly involves increasing aeration, which has been suggested by others,[12,13,17,19,20] or improving the conservation of cells between cycles of utilization. One option to maintain cell integrity and viability could be to use a glycerol solution or a mixture of glycerol and calcium chloride. The possibility of recycling *P. pastoris* KM71/cStp biomass to increase cStp yield suggests that other recombinant proteins expressed in this system could have their productivity increased by recycling of the biomass.

Conclusion

The recycling of recombinant *P. pastoris* biomass, as well as the immobilization of these recombinant strains for increased volumetric productivity of recombinant streptavidin,

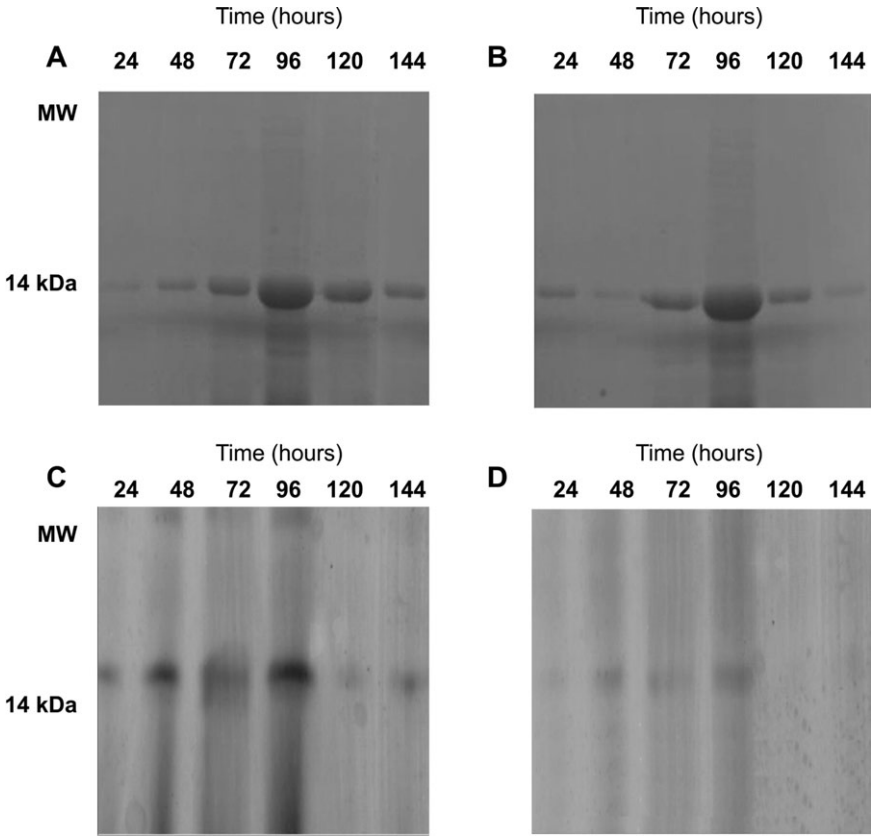

Figure 7. Comassie stained SDS-PAGE of cleared culture supernatant from *P. pastoris* KM71/cStp cultures.

Induction of free or calcium alginate-immobilized cells. (A) First induction cycle of immobilized cells. (B) Second induction of the immobilized cells. (C) First induction cycle of free suspended cell mass. (D) Second induction cycle of free suspended cell mass. Each lane was loaded with 20 μg of protein.

Table 3. HABA Assay for Streptavidin Detection

Sample	A1	A2	A1-A2
Deionized H_2O + HABA	0.196	0.194	0.002
Avidin (0.5 mg mL^{-1}) + HABA	1.048	0.128	0.868
Free cells (*P. pastoris* KM71/cSTP) + HABA	0.842	0.074	0.750
Immobilized cells (*P. pastoris* KM71/cSTP) + HABA	0.768	0.077	0.691
Control cells (*P. pastoris* KM71/cSTP) + HABA	0.163	0.144	0.019

Cleared supernatants from 96 h induced *P. pastoris* cultures were mixed with the HABA reagent and subject to absorbance reading at 500 nm. The assay was done with samples from free or immobilized recombinant cultures in parallel with the control (*P. pastoris* KM71/pPIC9). The values indicate the average of three measurements.

is shown in this work as an alternative for process development.

Together, these results show the expression of a functional cStp by the yeast *P. pastoris*. This novel strain could be used to express fusion proteins of industrial interest containing cStp as the functional molecule. The construction would allow the candidate protein to be separated and immobilized in one step in biotinylated columns. Our group is currently working on using cStp to immobilize pectinases and other enzymes of industrial interest (unpublished results). The data also contributes to the effort of improving protein production by the *P. pastoris* expression system. Moreover, studies to improve cell viability during biomass recycling to increase volumetric productivity should also be considered.

Acknowledgments

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