Folia Microbiologica (2021) 66:917–930
https://doi.org/10.1007/s12223-021-00894-w

ORIGINAL ARTICLE

Enhancing the expression of recombinant small laccase in *Pichia pastoris* by a double promoter system and application in antibiotics degradation

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Received: 26 March 2021 / Accepted: 22 June 2021 / Published online: 3 July 2021
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Abstract

Low-expression levels remain a challenge in the quest to use the small laccase (rSLAC) as a viable catalyst. In this study, a recombinant *Pichia pastoris* strain (rSLAC-GAP-AOX) producing rSLAC under both AOX and GAP promoters (located in two different plasmids) was generated and cultivated in the presence of methanol and mixed feed (methanol:glycerol). Induction with methanol resulted in a maximum laccase activity of 1200 U/L for rSLAC-GAP-AOX which was approximately 2.4-fold higher than rSLAC-AOX and 5.1-fold higher than rSLAC-GAP. The addition of methanol:glycerol in a stoichiometric ratio of 9:1 consistently improved biomass and led to a 1.5-fold increase in rSLAC production as compared to induction with methanol alone. The rSLAC removed 95% of 5 mg/L ciprofloxacin (CIP) and 99% of 100 mg/L tetracycline (TC) in the presence of a mediator. Removal of TC resulted in complete elimination of antibacterial activity while up to 48% reduction in antibacterial activity was observed when CIP was removed. Overall, the present study highlights the effectiveness of a double promoter system in enhancing SLAC production.

Introduction

Laccases are regarded as versatile biocatalysts owing to their broad substrate specificity enabling potential use in a wide range of applications (Xia et al. 2019). Bacterial laccases could prove to be useful alternatives to fungal laccases due to their intrinsic ability to tolerate high temperature and alkaline pH (Yadav et al. 2018). However, low production yield from native sources could present a limitation on potential industrial application (Dubé et al. 2008). Production of laccases in recombinant hosts such as *Pichia pastoris* could overcome this obstacle. Selection of suitable strong promoters as well as the signal sequence can be made during recombinant protein production which could direct the protein to culture media, thereby simplifying enzyme purification. Furthermore, optimising the expression conditions such as pH or temperature could also increase the enzyme yield (Antošová and Sychrová 2016).

Pichia pastoris, a methylotrophic yeast, is an excellent host in producing functionally active recombinant proteins due to its excellent protein secretory capability (Looser et al. 2014), ability to attain high cell densities using minimal media, stable integration of the gene of interest (GOI) (Wu et al. 2003), low secretion of native proteins and availability of strong promoters (Yadav et al. 2018). AOX1 and GAP promoter–based systems (P_{AOX1} and $P_{GAP,}$ respectively) are two of the easy to use expression systems commercially available for *P. pastoris*, where the former is methanol-inducible and, the latter, constitutively expressed (Gidijala et al. 2018). AOX1 is the most preferred promoter system due to the strong and tight regulation of the GOI cloned under its control, and this has allowed efficient production of several industrial enzymes (Öztürk et al. 2017). In addition, growth phase can be decoupled from protein production phase in the methanol inducible expression systems (Gidijala et al. 2018). Furthermore, strategies have been adopted to enhance the production level in P_{AOX1}-based systems such as codon optimisation, multicopy gene insertion, process optimisation, and co-expressing folding or secretion factors (Cámara et al. 2017).

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The laccase from *Streptomyces coelicolor* belongs to a group of two-domain multicopper oxidases. Most laccases contain three domains (domain 1, domain 2 and domain 3). However, the small laccase (SLAC) from *Streptomyces coelicolor* lacks the second domain (domain 2), hence its smaller size (~ 32 kDa) and the name small laccase. The SLAC is thermostable and has been applied in the decolorisation of dyes (Yadav et al. 2018), removal of phenolic pollutants (Yadav et al. 2021) and lignin degradation (Majumdar et al. 2014). However, its application seems to be limited by low enzyme yields as it is produced intracellularly in native hosts. In our previous study, the SLAC from *Streptomyces coelicolor* was heterologously expressed in *P. pastoris* under AOX1 promoter (Yadav et al. 2018). However, the slow growth rate and low enzyme productivity (mainly due to the use of methanol as both inducer and carbon source) are a bottleneck for effective enzyme production (Parashar and Satyanarayana 2016). Fed batch fermentations for heterologous protein expression in *P. pastoris* (under the control of AOX1 promoter) involve three phases, namely (i) glycerol batch phase (GBP), (ii) glycerol fed batch and (iii) methanol induction phase (MIP) (Kazemali et al. 2016). However, the first two phases that comprise only biomass generation and the final phase (MIP) is involved in the expression of recombinant protein (Hellwig et al. 2001; Holmes et al. 2009). The use of a double promoter system (constitutive and inducible promoters) could enhance heterologous enzyme production by facilitating enzyme production in all the three phases (Parashar and Satyanarayana 2016). It is known that high concentrations of methanol are toxic to *Pichia* cells (Yadav et al. 2018), and there is intrinsic correlation between growth and recombinant protein production; therefore, co-feeding with a different carbon source at non-repressing rates seems like a reliable approach in order to provide additional carbon source supporting growth and production levels (Canales et al. 2018). Therefore, the present investigation aims to enhance the extracellular expression of a recombinant small laccase (rSLAC) in *P. pastoris* by integrating the small laccase gene under the control of both constitutive and inducible promoters in a single clone. We also investigated the effect of different parameters on laccase production viz., pH, temperature and co-feeding with methanol:glycerol at shake flask level (50 mL). In addition, the crude rSLAC was applied in the degradation of two broad spectrum antibiotics, ciprofloxacin (CIP) and tetracycline (TC), as a model of their remediation from contaminated environments.

Materials and methods

Strains, vectors, chemicals and media

Escherichia coli DH5α and *P. pastoris* GS115 (Invitrogen) were used as the hosts for cloning and expression of laccase, respectively. *E. coli* ATCC 25,922 and *Staphylococcus aureus* ATCC 29,213 were used to determine antibacterial activity and were obtained from the Cape Peninsula University of Technology, South Africa, and are currently deposited in the stock culture collection in the Department of Biotechnology and Food Science, Durban University of Technology. These strains were maintained at 4 °C on nutrient agar slants and sub-cultured onto nutrient broth for 24 h prior to experiments. Plasmid, pGAPZαA (Invitrogen), was used for the expression of laccase.

Yeast extract peptone dextrose (YPD) and yeast extract peptone (YP) were used to culture the methylotrophic yeast, *P. pastoris*, and were prepared according to the manual of EasySelect Pichia expression kit (Invitrogen). pET28a(+) (Novagen) was used as a source of the kanamycin resistance gene used in this study. Low salt LB (supplemented with 25 µg/mL zeocin) was used for the propagation of DH5α competent cells. Nutrient broth (NB) was used to grow *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. CIP, TC, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and G-418 (Geneticin) were purchased from Sigma-Aldrich. Stock solutions of CIP (0.1 mg/mL) and of TC (0.2 mg/mL) were prepared and stored at 4 °C. The restriction enzymes (*Eco*RI, *Nco*I, *Nde*I, *Sac*I, *Avr*II and *Xba*I) were procured from New England Biolabs and primers (Table S1) from Integrated DNA Technologies (IDT).

Construction of pGAPKαA vector with unique selection marker

Primers P1 (pGAPαA_F) and P2 (pGAPαA_R) with flanking regions of an *Nco*I and an *Nde*I restriction sites, respectively, were used to amplify pGAPZαA vector lacking zeocin encoding region. A unique *Nde*I site was inserted in pGAPZαA to clone in the kanamycin resistance encoding region. Kanamycin resistance encoding sequence (816 bp) was amplified using primers P3 (Kan_F) and P4 (Kan_R) from pET28a(+) vector, allowing for the introduction of *Nco*I and *Nde*I restriction sites, respectively. All amplifications were carried out in a GeneAmp PCR System 9700, Applied Biosystems using Phusion High-Fidelity DNA Polymerase in a 50-µL reaction mixture with the set PCR conditions (in Supplementary file).

Construction of pGAPKαA-SLAC

Primers (P5 and P6; Table S1) and conditions used for the amplification of the *SLAC* gene were used as previously described (Yadav et al. 2018). pGAPZαA vector was modified to pGAPKαA according to Parashar and Satyanarayana (2016), whereby the gene conferring zeocin resistance was replaced by the kanamycin resistance gene (816 bp). Restriction digestion of the PCR product (SLAC) and pGAPKαA were carried out with *Eco*RI and *Xba*I for 1 h at 37 °C in the

presence of Cut Smart buffer and the products purified by GeneJet purification Kit (Thermofischer Scientific). Ligation of digested products was carried out for the generation of pGAPKαA-*SLAC* (Fig. S1a) as previously described by Yadav et al. (2018). The ligation mixture was transformed into *E. coli* DH5α competent cells, and the transformants obtained were confirmed by colony PCR and double digestion, as well as followed by automated DNA sequencing.

Transformation of *P. pastoris*

The electrocompetent *P. pastoris* cells were prepared and were transformed with constructs according to the manufacturer's protocol (Invitrogen). A negative control was also prepared simultaneously by transforming WT *P. pastoris* with an empty vector (without SLAC gene). *SLAC*-GAP was generated by transforming wild type *P. pastoris* cells with linearised pGAPKαA-*SLAC* (linearised by *Avr*II). The transformed cells were plated onto YPD agar (g/L): yeast extract 10, peptone 20 and dextrose 20 and agar 20, containing varying concentrations (200–400 µg/mL) of G-418 (an aminoglycoside antibiotic) and grown at 30 °C for 72 h. Positive transformants harbouring SLAC gene were identified using colony PCR strategy employing gene specific primers as described by EasySelect™ Pichia Expression Kit (Invitrogen). Since the ability of the transformants to tolerate high concentration of antibiotic correlates with the gene copy number, clones capable of growing at the highest concentrations of geneticin were selected for shake flask screening.

Shake flask expression studies

For this, a single colony was picked and inoculated in 10 mL of YPD medium in a 250-mL flask and the culture was grown at 30 °C and shaking at 250 rpm until the culture reached an OD_{600} of 2–6 (in about 16–18 h). Then the fresh YPD medium (50 mL) was inoculated with 1% (v/v) seed culture and incubated at 30 °C while shaking at 250 rpm for 7 days. Aliquots were drawn every 24 h and centrifuged (1500 × g, 10 min) to separate the culture supernatant from the cell biomass. Thereafter, 1 mM $CuSO_4$ was added to the supernatant (to restore enzyme activity) and a quantitative laccase assay was performed. The best clone shortlisted from screening had laccase activity of **234 U/L**.

Further, to generate *SLAC*-GAP-AOX strain, pGAPKαA-*SLAC* was made competent (as described by Invitrogen) and transformed by pPICZαA-*SLAC* (linearised by *Sac*I) vector as described by Yadav et al. (2018). The transformed cells were plated on YPD agar supplemented with 1.0 M sorbitol containing zeocin (100, 250, 500 or 1000 µg/mL) to select for high copy number of AOX-SLAC integrants. The plates were incubated at 30 °C for 72 h to visualise single distinct colonies. Colony PCR was performed to identify positive clones. Further to confirm the integration of both the constructs we plated the obtained clone on a YPD medium with both selection markers (i.e. geneticin and zeocin). Furthermore, small-scale studies (in shake flasks) were carried out to screen for the hyper-producer of SLAC. For induced expression under AOX1 promoter, a single colony was picked and transferred to 10 mL of YPD broth and cultivated at 30 °C (200 rpm) until the OD_{600} of 5–6 was reached. Thereafter, the cells were harvested by centrifuging at 1500 × g for 5 min and washed twice with autoclaved Milli-Q to remove any residual dextrose. Cells were then resuspended in YP medium till OD_{600} reached 1.0 followed by methanol addition after every 24 h interval to maintain induction. Samples were collected after every 24 h and assayed for laccase activity. Strains exhibiting highest activity (also called hyper-producers) were shortlisted for further optimisation experiments.

Determination of laccase activity

Laccase activity was determined using ABTS as a substrate, as described previously (Yadav et al. 2018). Oxidation of ABTS (1 mM) was measured at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) in 20 mM acetate buffer (pH 4.0). One unit of laccase activity (U) was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute (Jin et al. 2016).

Optimisation for rSLAC production in shake flasks

pH and temperature

Optimum temperature was determined by growing the recombinant strains at different cultivation temperatures (16, 20, 24, 28 and 32 °C), and the optimum pH for laccase production was determined over the pH range pH 4.0–8.0. Experiments were carried out in triplicate, and results are shown as the mean ± standard deviation (SD) of three independent experiments.

Mixed feed fermentation

The influence of a mixed feed (glycerol:methanol) was investigated in the production of rSLAC by the *Pichia* strain harbouring rSLAC-AOX-GAP and rSLAC-AOX. A mixture of methanol and glycerol was added after every 24 h in different stoichiometric concentrations (100:0, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100) to a final concentration of 2% (v/v) (optimisation data not shown) in order to maintain induction.

Sampling was performed every day to determine cell growth (OD_{600}) and the expression level of rSLAC.

Degradation and detoxification of CIP and TC by rSLAC

Enzymatic transformation of CIP and TC in aqueous solution

For preliminary set of experiments, the reaction mixture (1 mL) consisted of individual antibiotics (CIP at 5 mg/L; TC at 50 mg/L) in Tris–HCl buffer (50 mM, pH 8.0) and a mediator, acetosyringone (AS) (4 mM). Typically, mediators are used in conjunction with laccase to enhance transformation of recalcitrant compounds (Murugesan et al. 2010). Therefore, a natural mediator, acetosyringone, has been included in the experiments to facilitate the transformation of CIP and TC. The reaction was started by adding rSLAC (0.4 U) and was incubated in the dark with mild shaking (50 rpm) at 37 °C. A parallel control reaction was prepared that consisted of antibiotic with mediator to rule out the degradation of antibiotic by abiotic factors. Additionally, a negative control reaction was set up by adding culture supernatant obtained from *Pichia* harbouring vector alone (without laccase gene), thereby attesting that the observed transformation of antibiotics is due to rSLAC.

Optimisation of reaction conditions in enzyme-catalysed systems can accelerate the degradation efficiency of antibiotics. Therefore, to examine the optimum conditions for degradation of model antibiotics (TC and CIP) by rSLAC, we investigated degradation of different initial concentrations of antibiotics (CIP 2.5–10 mg/L; TC 50–200 mg/L), at various pH levels (4.0–8.0) and AS concentrations (1–4 mM).

For time course studies, reactions were carried out under optimum conditions and at pre-selected time intervals, samples were withdrawn and stored at − 20 °C until analysis. The residual concentration of CIP and TC in the reaction mixtures was analysed by HPLC as described below.

Removal efficiency was calculated according to Sun et al. (2017) as follows:

$$\text{Removal } \% = \left(C_0 - C_t\right)/C_0 \times 100$$

where C_0 (mg/L) corresponds to the concentration of antibiotic at time 0 and C_t (mg/L) corresponds to the residual concentration (after rSLAC treatment) of antibiotic at time t.

Quantitative analysis of antibiotics and its degradation by HPLC

Antibiotics and their degradation products were analysed using reversed phase chromatography on a Shimadzu HPLC system from Dionex (MA, USA) equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector. Chromatographic separation was achieved on a C18 column (Sunfire, Waters). The mobile phases used for analysis were 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.5 mL/min. The injection volume was 10 µL, and an oven temperature of 25 °C was used. Isocratic elution was used with 15% B for 30 min, and the degradation products were quantified at 254 nm.

Assay for bacterial growth inhibition

The residual antibacterial activities of the degradation products of CIP and TC (after rSLAC treatment) were evaluated as percentage growth inhibition or qualitatively using well diffusion assays.

A well diffusion method was employed to determine the antibacterial activity of the samples according to Pan et al. (2018) with slight modifications. A Gram-positive (*S. aureus,* ATCC 29213) and a Gram-negative (*E. coli*, ATCC 25922) bacterial strains were selected as representative microorganisms for this study. Briefly, 0.1 mL of each culture (OD_{600} of 2.0) was spread onto nutrient agar plates. Thereafter, wells were punctured into the agar surface using a sterile well borer. Each well was spiked with 40 µL of treated samples followed by an overnight incubation at 37 °C. Two sets of controls were set-up where the first control consisted of the untreated antibiotic solution (2.5 mg/L of CIP and 100 mg/L of TC), while the second set of controls consisted of solvents used for preparing stock solutions of CIP and TC, i.e. double-distilled water (for TC) and DMSO (for CIP), to rule out the effect of solvent. The zone of inhibition (indicating antibacterial activity) was measured in millimetres, and each experiment was repeated in triplicate; the mean values ± SD are reported.

Growth inhibition tests were also conducted using *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 as previously described by Sun et al. (2017) with minor modifications. Briefly, the strains were cultured in nutrient broth (NB) at 37 °C with shaking at 200 rpm for 24 h. Subsequently, standard antibiotic solutions (2.5 mg/L of CIP and 100 mg/L of TC) and the treated reaction mixtures of CIP and TC (40 µL) were added into the NB medium along with the bacterial suspension (100 µL) and incubated under the same conditions. After 24 h incubation, absorbance of the samples was measured which were then converted into percent growth inhibition according to the equation:

$$\text{Growth inhibition } \% = \left(OD_{600C} - OD_{600T}\right)/OD_{600C} \times 100,$$

where OD_{600C} corresponds to the OD of the control sample (i.e. *E. coli* or *S. aureus* culture) and OD_{600T} corresponds to the OD of the test sample consisting of the rSLAC-treated solutions of CIP and TC.

Results and discussion

Development of recombinant *Pichia* strain with double promoter for heterologous production of rSLAC

In our previous work, the SLAC gene from *S. coelicolor* was expressed in *P. pastoris* under the control of an inducible promoter P_{AOX1}. With optimum fermentation conditions, enzyme activity reached 500 ± 10 U/L (Yadav et al. 2018). Even though the production level was higher than some earlier reports (Lu et al. 2013; Chen et al. 2015), intense efforts are needed to further enhance the production levels of SLAC in *P. pastoris*. Several researchers have investigated the use of double promoter to enhance the transcriptional level of industrial important enzymes/proteins such as human granulocyte–macrophage colony-stimulating factor ((hGMCSF), α-Amylase, β-mannanase (acidic and alkaline), lipase, phytase (Öztürk et al. 2017) and κ-carrageenase (Yu et al. 2020). Usage of double promoters expression system has resulted in the enhancement of expression of various recombinant enzymes 1.1- to sevenfold (Öztürk et al. 2017) when compared to single promoter systems. It is worth noting that there is no report on the use of double promoters in enhancing the production levels of laccase in *P. pastoris*. This prompted us to explore the use of double promoter strategy in enhancing expression levels of SLAC in *P. pastoris*. To accomplish this, the vector pGAPZαA was modified by incorporating the Kanamycin resistance gene (Fig. S1). The engineered pGAPZαA now lacks the zeocin encoding region and confers resistance against kanamycin in bacteria as well as geneticin in yeast. Two *Pichia* strains were generated: pPICZαA-*SLAC* (from our previous study, Yadav et al. 2018) and pGAPKαA-*SLAC* producing SLAC under AOX1 and GAP promoters, respectively. Furthermore, the recombinant *Pichia* strain harbouring pGAPKαA-*SLAC* was made competent (by electroporation) and transformed for the second time with linearised pPICZαA-*SLAC* to generate rSLAC-GAP-AOX capable of synthesising SLAC in the presence of both methanol and glycerol. This clone showed resistance towards both the marker genes (G-418 and zeocin), indicating successful integration of both plasmids.

rSLAC production by recombinant *Pichia pastoris* (rSLAC-GAP-AOX) strain in shake flask cultures

The profile for cell growth and rSLAC production from the recombinant *Pichia* strains harbouring single and double promoters is shown in Fig. 1. Maximum rSLAC expression level was achieved after the 5th day of cultivation (1200 U/L) which was 2.4-fold higher than the single promoter system (rSLAC-AOX). In addition, there was also an increase in the cell growth of the recombinant *Pichia* strain with rSLAC-GAP-AOX (Fig. 1). It is worth noting that the production levels were found to be higher than levels of other bacterial laccases such as from *Bacillus licheniformis* (227.9 U/L) (Lu et al. 2013), *Bacillus amyloliquefaciens* LC02 (379.7 U/L) (Chen et al. 2015), *Bacillus subtilis* WD23 (891.2 U/L) (Fan et al. 2015) and a D500G mutant of *Bacillus licheniformis* LS04 laccase (347 U/L) (Wang et al. 2017) expressed in *P. pastoris*. This indicates that using double promoter system is an efficient strategy as compared with the standard expression in *P. pastoris*.

Optimisation of cultivation parameters

pH and temperature

Cultivation parameters such as medium components, pH and temperature have been known to significantly affect protein production (Wang et al. 2017). Improper culture conditions could result in cell lysis and decrease in viability of *P. pastoris* cells (Wang et al. 2009). *P. pastoris* can tolerate a broad range of pH and therefore the expression of rSLAC was evaluated over a pH range of 4.0 to 8.0. Although *P. pastoris* growth was not greatly affected by pH, laccase expression increased with an increase in the pH of the medium (up to pH 7.0) (Fig. 2a). The results also indicate the stability of rSLAC over a broad pH range. However, low activity at acidic pH (i.e. pH 4.0) could possibly be attributed to the activation of acidic proteases which could have resulted in the loss of enzyme stability. This is consistent with other reports which also documented the proteolytic degradation of enzymes at low pH (Inan et al. 1999; Soden et al. 2002). Alternatively, protease-deficient strains *P. pastoris* SMD1168H have been used in order to alleviate proteolysis of recombinant protein; however, they have lower growth rates and take longer duration to express (Wang et al. 2015) when compared to the wild type strains. In addition, it is worth mentioning that optimal pH of the medium can vary for different recombinant proteins.

The optimal cultivation temperature for *P. pastoris* is usually between 28 and 30 °C. Laccase expression generally increased with increase in cultivation temperature (Fig. 2b). An optimum temperature of 28 °C was recorded for both rSLAC-AOX and rSLAC-GAP-AOX strains; a slightly lower expression level was observed at 32 °C. In a previous study, temperature higher than 32 °C negatively affected protein expression and resulted in cell death (Karbalaei et al. 2020). Lower laccase expression at 32 °C could also be due to the induction of endoplasmic reticulum (ER)-phagy which can in turn result in yeast cell death

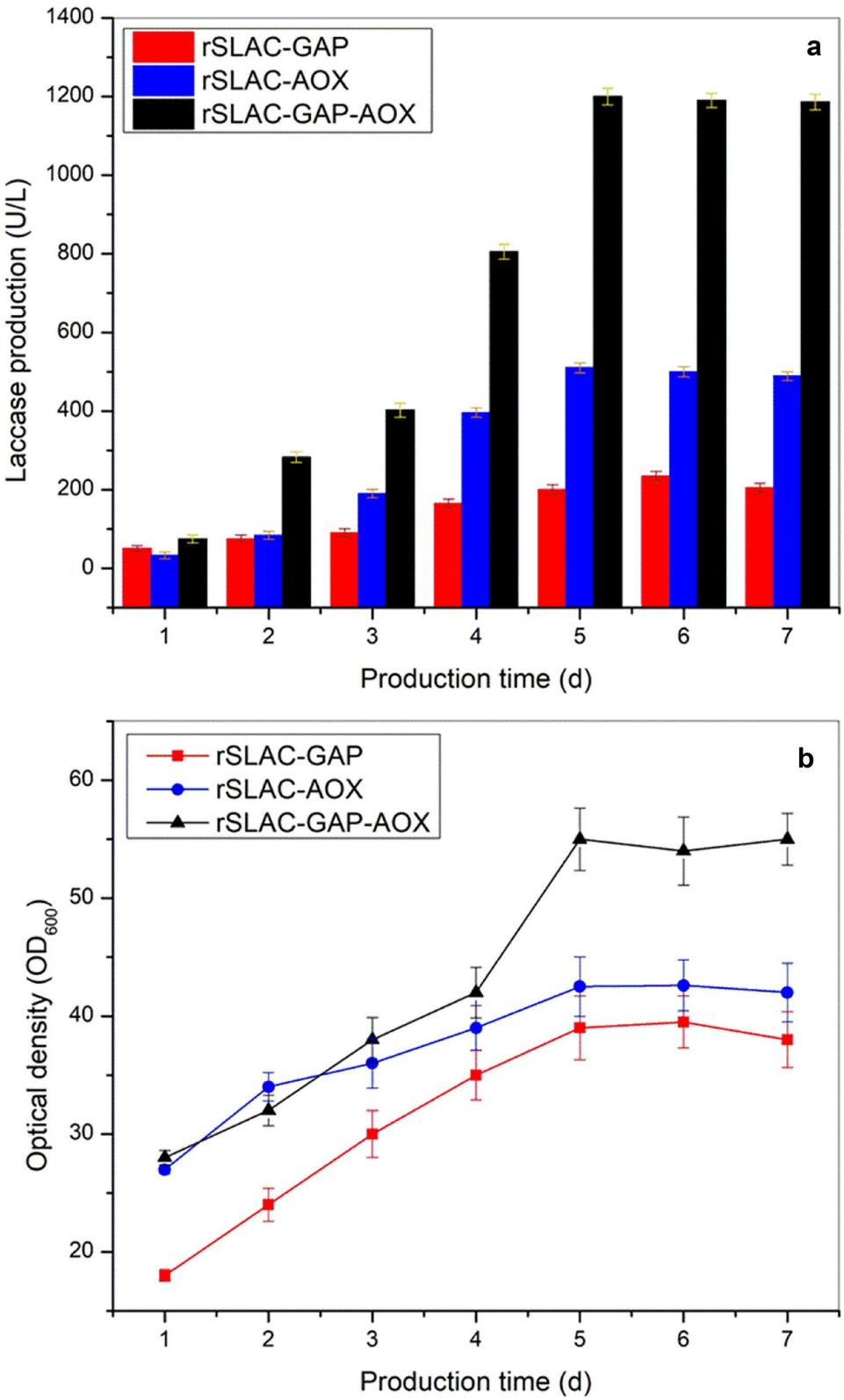

Fig. 1 Time course of (**a**) rSLAC production and (**b**) cell growth by the recombinant *Pichia* strain harbouring rSLAC-GAP, rSLAC-AOX and rSLAC-GAP-AOX. The recombinant *Pichia* strain was cultivated in YP medium and was supplemented with 2% (v/v) methanol after every 24 h. Data are presented as the mean ± standard deviation (SD) of triplicate determinations

(Zhong et al. 2014). After 5 days of cultivation, rSLAC production level at 28 °C was approximately 3.2 times higher than at 16 °C. Clearly, lowering the cultivation temperature did not favour the expression of rSLAC in *P. pastoris* which could be ascribed to the low biomass production at 16 °C (data not shown).

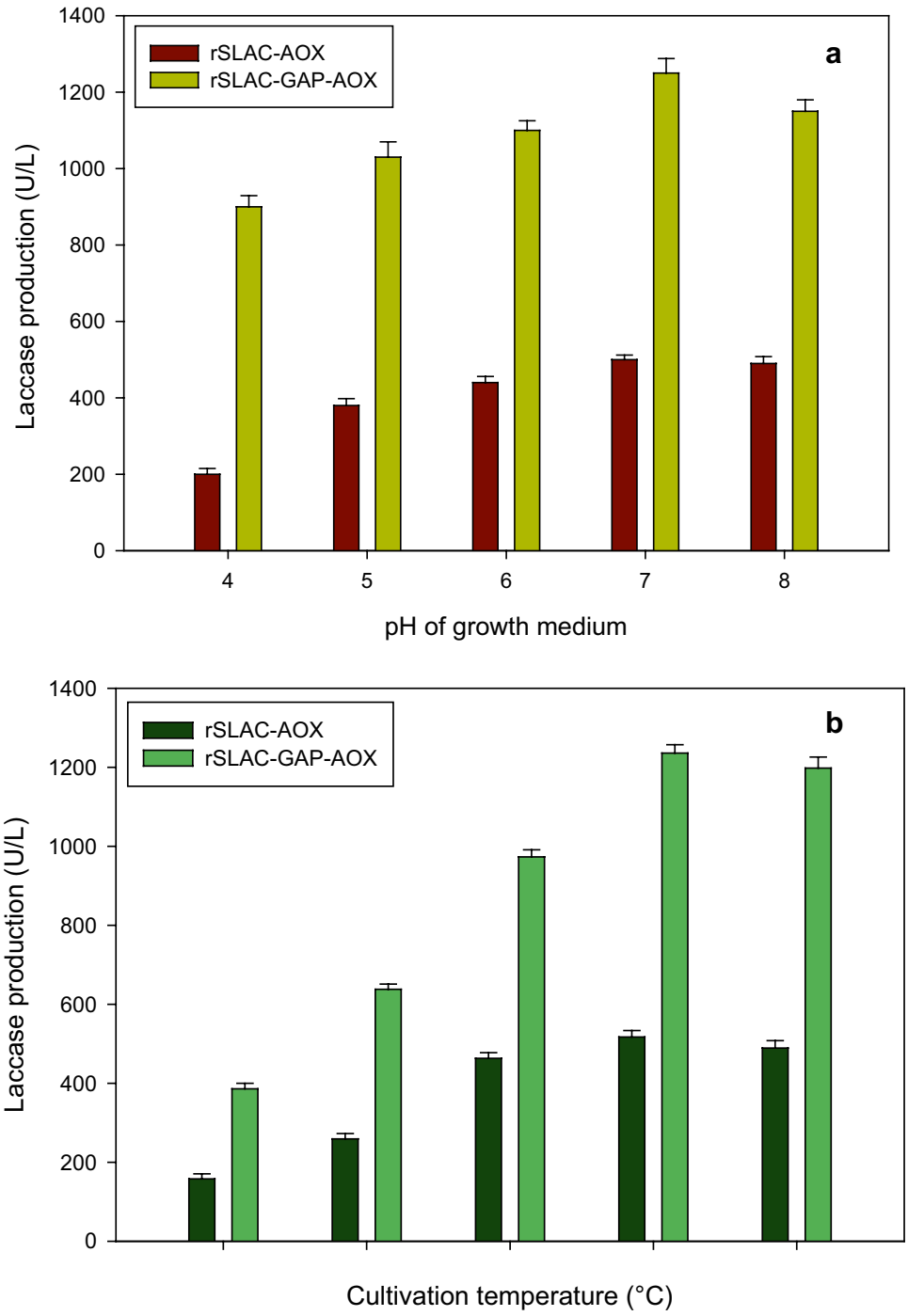

Fig. 2 Optimisation of (**a**) the pH of the medium used and (**b**) cultivation temperature for the production of laccase from rSLAC-GAP-AOX and rSLAC-AOX *Pichia* strains, respectively. The results are represented as an average value of three separate experiments ± SD

Mixed feed

The rSLAC-GAP-AOX strain produced higher titres of laccase when the production medium (YP) was supplemented with a mixed feed of methanol:glycerol as compared to rSLAC-AOX. Among different ratios of methanol:glycerol tested, maximum production level (1800 U/L) was achieved with a 9:1 ratio of methanol:glycerol (Fig. 3a). This was found to be 1.1-fold higher than the rCotA expressed in *P. pastoris* (1648 U/L in 24 days) after sorbitol inclusion and pH adjustment of the medium (Wang et al. 2015). In the case of the strain harbouring rSLAC-AOX, maximum enzyme production was obtained with 100% methanol induction. When a single promoter system was supplied with a mixed feed, laccase production was negatively affected (Fig. 3b). This is possibly due to the inability of this system to utilise glycerol, which in turn represses the AOX1 promoter (Parashar and Satyanarayana 2016) resulting in repression of rSLAC transcription.

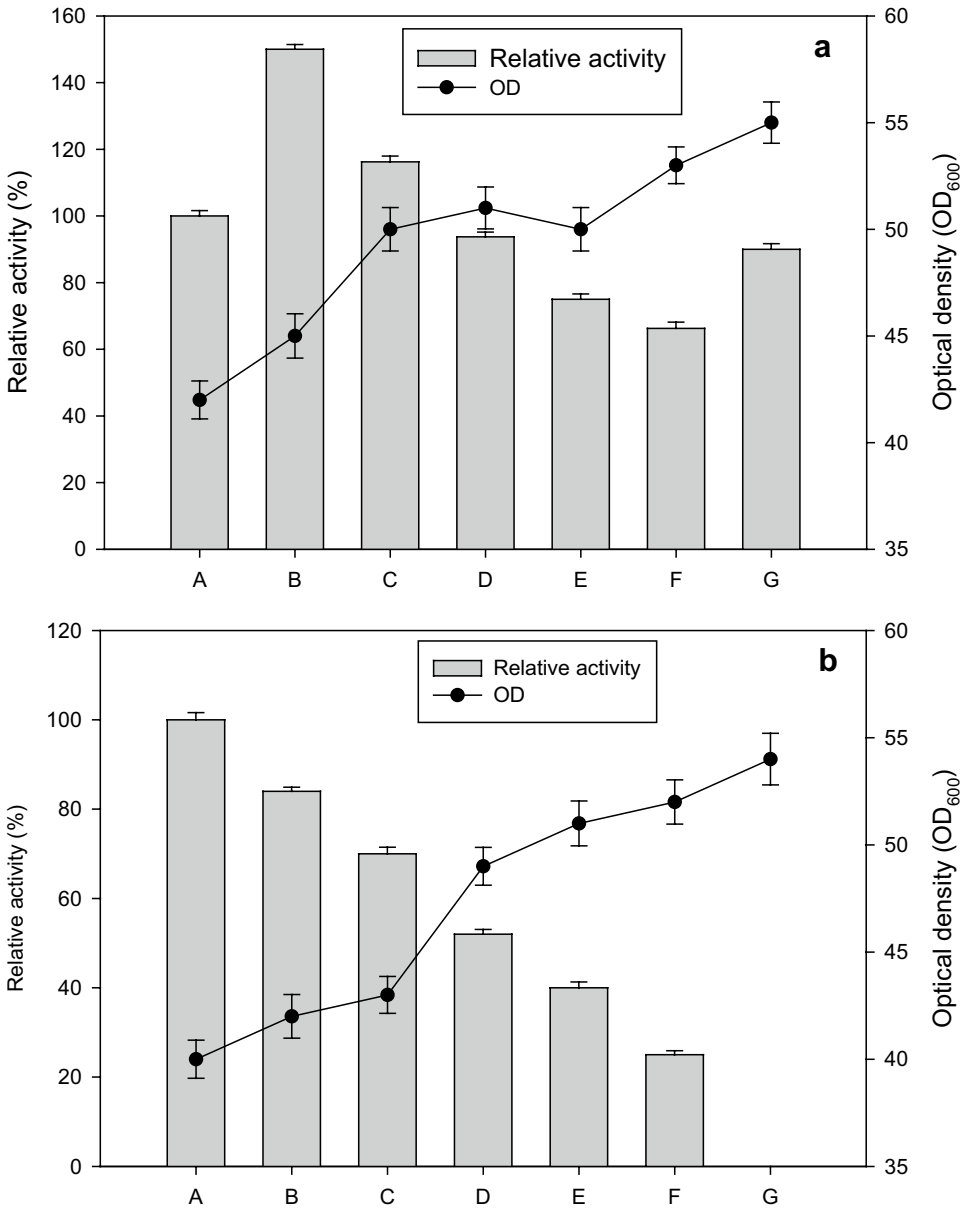

Fig. 3 Effect of mixed feed cultivation on cell biomass (—●—) and rSLAC expression by a *Pichia* strain harbouring (**a**) rSLAC-GAP-AOX and (**b**) rSLAC-AOX, cultivated in YP medium. A, B, C, D, E, F and G denote the 2% (v/v) mixed feed of methanol:glycerol (100:0, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100, respectively)

Even though methanol can be utilised by *P. pastoris* as a sole energy source (Parashar and Satyanarayana 2016), excessive methanol can negatively impact cell growth due to the accumulation of toxic products of methanol oxidation such as formaldehyde and hydrogen peroxide (Zhang et al. 2003). In addition, protein overexpression in a methanol-based system interferes with carbon utilisation. It is also worth noting that higher product yields and improved cellular growth might not be achieved by simply increasing methanol concentration (Panula-Perälä et al. 2014). However, to address these problems, a co-feeding strategy has frequently been explored. Addition of co-substrates like glycerol, glucose, sorbitol and mannitol can improve cellular growth and enhance enzyme titres (Yang and Zhang 2018). Therefore, the growth rate of *Pichia* cells is lower in methanol as compared to glycerol. However, low enthalpy of combustion of glycerol leads to lower heat production and low oxygen consumption rate during growth (Parashar and Satyanarayana 2016). In addition, the use of multiple substrates also generates NADH/ATP, owing to the shift in energy metabolism from formaldehyde dissimilation to the TCA cycle. This ultimately reduces metabolism damage of cells and relieves energy supply burden (Yang and Zhang 2018; Liu et al. 2019). In high cell density fermentation with *P. pastoris,* maintenance of temperature and adequate supply of oxygen represent technical limitations. Therefore, in such a case, supplementing with methanol:glycerol could enhance cell biomass and process productivity (Parashar and Satyanarayana 2016). Several authors have reported positive influence of mixed feed of glycerol and methanol on cell culture viability and

rate of protein production (McGrew et al. 1997; Woo et al. 2004; Parashar and Satyanarayana 2016). It is evident from the present study, that the mixed feed positively influenced cell biomass production which, however, is not consistent with rSLAC production (Fig. 3a, b). This could possibly be attributed to the well known repression of AOX1 promoter by residual glycerol (Cereghino et al. 2002; Canales et al. 2018).

Degradation and detoxification of antibiotics

Degradation of CIP and TC by rSLAC

In an enzyme-catalysed reaction, pH plays a significant role in the transformation of contaminants (Sun et al. 2017). At the different pH levels tested, TC degradation was not greatly affected by pH. However, in the case of CIP, removal efficiency was found to be pH dependent. At pH 4, the degradation rate was low (20%). However, with an increase in pH (5 to 8), the removal percentage improved to 95%. From Fig. 4a, it is clear that the degradation rate for both antibiotics improved with an increase in the pH of the reaction medium and a maximum degradation level of 95 and 100% was achieved for 5 mg/L of either of the antibiotics (CIP and TC), respectively, at pH 8. This is in contrast with other reports where optimal pH for degradation of antibiotics was found to be from low to neutral range (i.e. pH range of 4–7) (Suda et al. 2012; De Cazes et al. 2014; Sun et al. 2017; Yang et al. 2017). The result seems promising as the reported pH of urban wastewater and hospital effluents is in the alkaline range (Cruz-Morató et al. 2013, 2014).

An investigation of the rSLAC-mediated degradation of CIP and TC revealed that rSLAC alone could degrade 16%

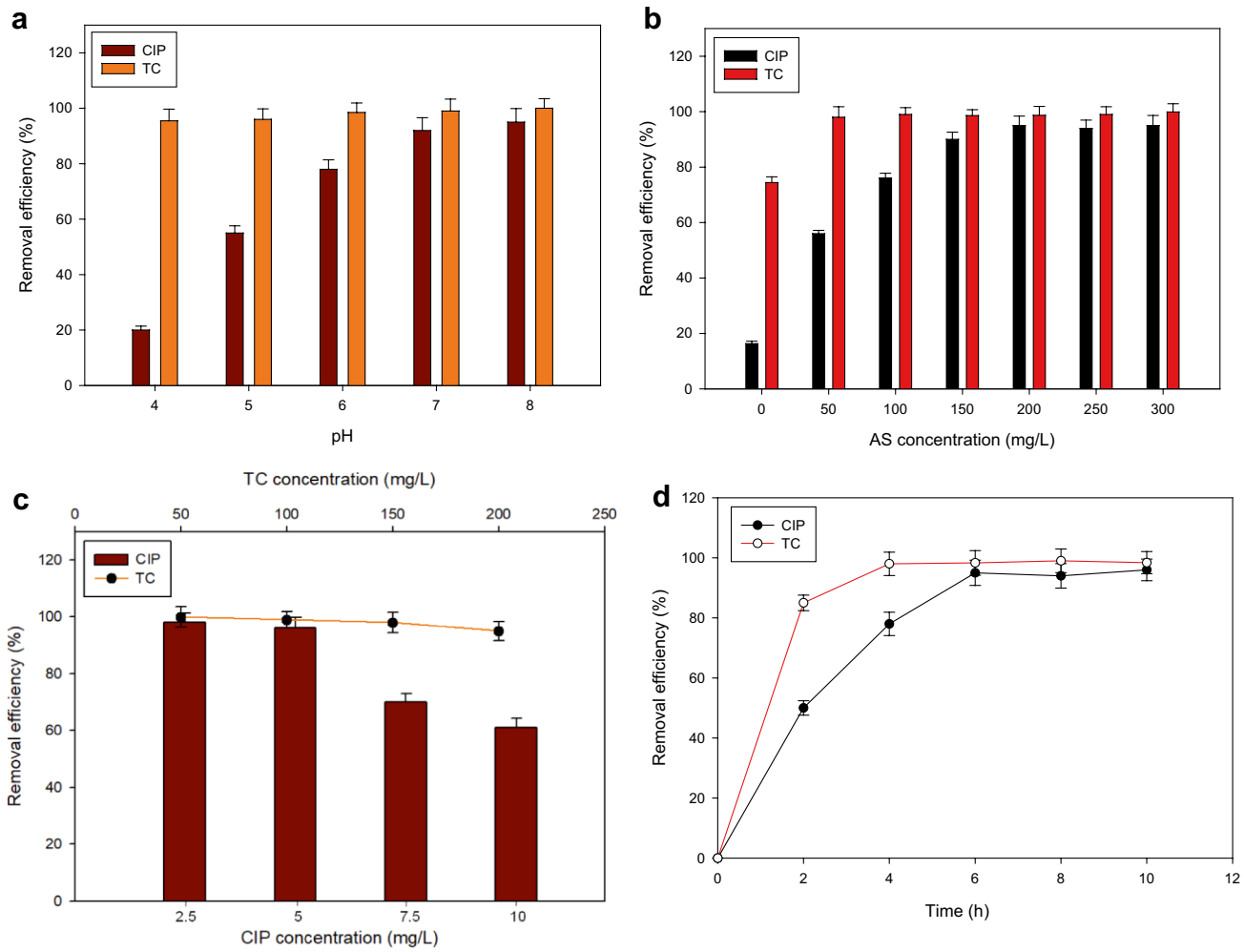

Fig. 4 Transformation of ciprofloxacin (CIP) and tetracycline (TC) by rSLAC AS-mediated reaction under varying conditions (**a**–**c**) for a 12-h incubation. (**a**) [CIP] = 5 mg/L, [TC] = 50 mg/L; [AS] = 200 mg/L, [rSLAC] = 0.4 U/mL, pH 4.0–8.0; (**b**) [CIP] = 5 mg/L, [TC] = 50 mg/L; [rSLAC] = 0.4 U/mL; [AS] = 0–300 mg/L, [pH] = 8.0; (**c**) [CIP] = 2.5–10 mg/L, [TC] = 50–250 mg/L; [rSLAC] = 0.4 U/mL, [AS] = 200 mg/L, [pH] = 8.0; (**d**) time course degradation of the antibiotics: [CIP] = 5 mg/L, [TC] = 100 mg/L, [AS] = 200 mg/L, [rSLAC] = 0.4 U mL^{-1}, [pH] = 8.0. Standard deviations are depicted by error bars and are based on experiments performed in triplicate

and 74% of 5 mg/L of CIP and 5 mg/L TC, respectively. The difference in the degradation efficiency of these antibiotics could be due to the difference in their structure, where TC has a phenol structure which is not present in CIP (Fig. S2). However, in order to enhance the oxidative potential of rSLAC, a mediator was added to the reaction. Upon laccase oxidation, mediators form radicals that act as an electron shuttle between the substrate and laccase, thereby expanding the substrate range of the laccase (Cruz-Morató et al. 2014). However, in order to develop a sustainable approach of remediation, we used a natural mediator, acetosyringone (AS) in the degradation of the antibiotics. Furthermore, we optimised the concentration of AS (0–300 mg/L) for the degradation of 5 mg/L of CIP and 50 mg/L of TC and achieved ≥ 95% degradation of CIP and TC at a 200 mg/L concentration of AS (Fig. 4b). In another study, quite a high concentration of artificial mediator, 1-hydroxybenzotriazole (400 mg/L) was used to achieve complete degradation of TC (Sun et al. 2017). Results obtained here suggest that rSLAC coupled with AS may be capable of remediating CIP and TC from contaminated environments. This is important since fluoroquinolones and tetracyclines are persistent micropollutants. The concentration of these drugs usually varies from nanograms to micrograms per litre in environment; however, in some cases, effluents released from hospitals or pharmaceutical industries may contain levels of up to milligrams per litre (Becker et al. 2016). Notably, the concentrations of CIP were found to be 67–119.2 ng/L (Pena et al. 2010), 42.8 µg/L (Lien et al. 2016) and 32–99 µg/L (De Lima Perini et al. 2017) from hospital and municipal wastewaters, whilst concentrations of 0.11 µg/L from US surface waters (Lindsey et al. 2001), 400 ng/L from swine confinement facilities (Krapac et al. 2005) and 2.37 µg/L from sewage treatment plants (Deblonde et al. 2015) have been reported for TC. The presence of antibiotics in aquatic environments stimulates the occurrence of antibiotic resistance genes, which causes severe epidemiological consequences worldwide (Zhang and Zhang 2011; Sprenger and Fukuda 2016; Sun et al. 2017).

Figure 4c shows that the transformation of CIP decreases from 98 to 60% as the initial CIP concentration increases from 2.5 to 10 mg/L. However, in the case of TC, transformation decreased from 100 to 95% when the concentration was increased from 50 to 200 mg/L. It is worth noting that only 0.4 U rSLAC was used to degrade ≥ 95% of 5 mg/L of CIP and 200 mg/L of TC, indicating a high catalytic efficiency of rSLAC as also supported by our earlier report (Yadav et al. 2018). Comparatively, high dosage, 5 U of fungal laccase, was used to achieve complete degradation of 50 mg/L of TC (Sun et al. 2017).

Time course for degradation of both antibiotics showed that maximum degradation (i.e. ≥ 95%) was achieved in 6 h (Fig. 4d). Remarkably, degradation of 50% of CIP and 85% of TC was achieved within 2 h.

HPLC analysis of degradation products

Fig. 5 shows the chromatograms of untreated (reaction without rSLAC) and treated (reaction with rSLAC) CIP and TC solutions. CIP, TC and AS were detected at retention times of 5.0, 6.2 and 28.8 min, respectively. However, after rSLAC treatment, the peak of CIP was significantly reduced indicating a low residual concentration of CIP after treatment with laccase. A number of transformation products were observed indicating degradation of CIP. On the other hand, TC treated samples showed complete depletion of TC (tR 6.2 min), along with the appearance of at least five transformation products.

Residual antibacterial activity of the untreated and treated antibiotics

Assessment of the antibacterial activity of the transformation products generated after treatment is vital in order to evaluate the efficacy of the treatment method. According to several previous reports, degradation does not always lead to complete mineralisation, resulting in residual antibacterial activity of the degraded parent compounds (De Bel et al. 2009; Kusari et al. 2009; Marco-Urrea et al. 2009; Sturini et al. 2012; Pan et al. 2018). Therefore, we assessed the antibacterial activity of the untreated and rSLAC-treated solutions of CIP and TC by a quantitative (growth inhibition) and qualitative (well diffusion) analysis (Fig. 6a, b). It is evident that the transformation products of CIP and TC was accompanied by considerable reduction in the antibacterial activity (48% reduction in the case of CIP and 99% reduction in the case of TC) as compared to their untreated parent compounds (Fig. 6a). In rSLAC-treated samples of CIP, a lower growth inhibition was observed which could be due to the residual CIP in the reaction mixture or due to the formation of toxic transformation product(s). Conversely, growth inhibition was completely removed in case of treated TC samples indicating complete removal of antibacterial potency of TC after rSLAC treatment.

In well diffusion assays (Fig. 6b), the diameter of the halo around wells reflects the antibacterial potential of the compound(s). Figure 6b shows that antibiotic controls of CIP and TC formed distinct zones of inhibition against both Gram-positive *S. aureus* and Gram-negative *E. coli*. Solvents, DMSO (in case of CIP) and distilled water (in case of TC), did not affect the growth of the reference microorganisms (Fig. 6b). However, in the rSLAC-treated CIP samples, the diameter of the zone of inhibition was reduced from 26 to 12 mm in the case of *E. coli* and from 16 to 12 mm in the case of *S. aureus*. However, no inhibition zone was observed for the rSLAC-treated TC samples, indicating complete reduction of antibacterial potency of TC via rSLAC treatment in 6 h. Therefore, it was evident from both the assays that rSLAC

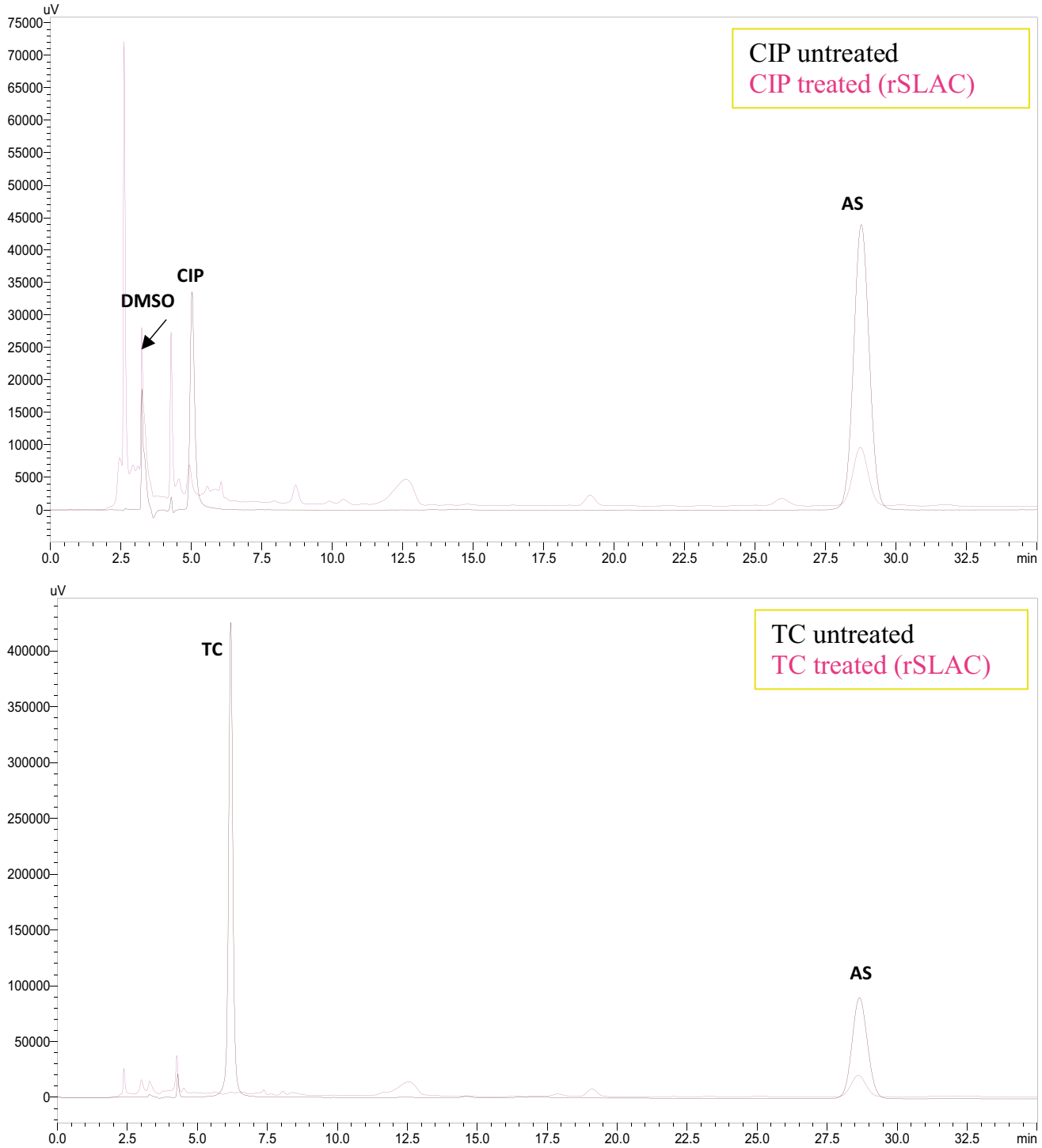

Fig. 5 HPLC chromatograms of ciprofloxacin (CIP) (5 mg/L) and tetracycline (TC) (100 mg/L) and their transformation products after rSLAC treatment. The presence of the mediator, acetosyringone (AS), is also indicated on the chromatograms

degraded the antibiotics and reduced their antibacterial activities. In addition, the results obtained from the qualitative well diffusion assays were consistent with those from the quantitative percentage growth inhibition studies. Though our results report growth inhibition with *E. coli* and *S. aureus*, a similar trend can also be expected with other bacteria as well, since the mechanism of action of CIP and TC remains the same for all susceptible bacteria (Paul et al. 2010).

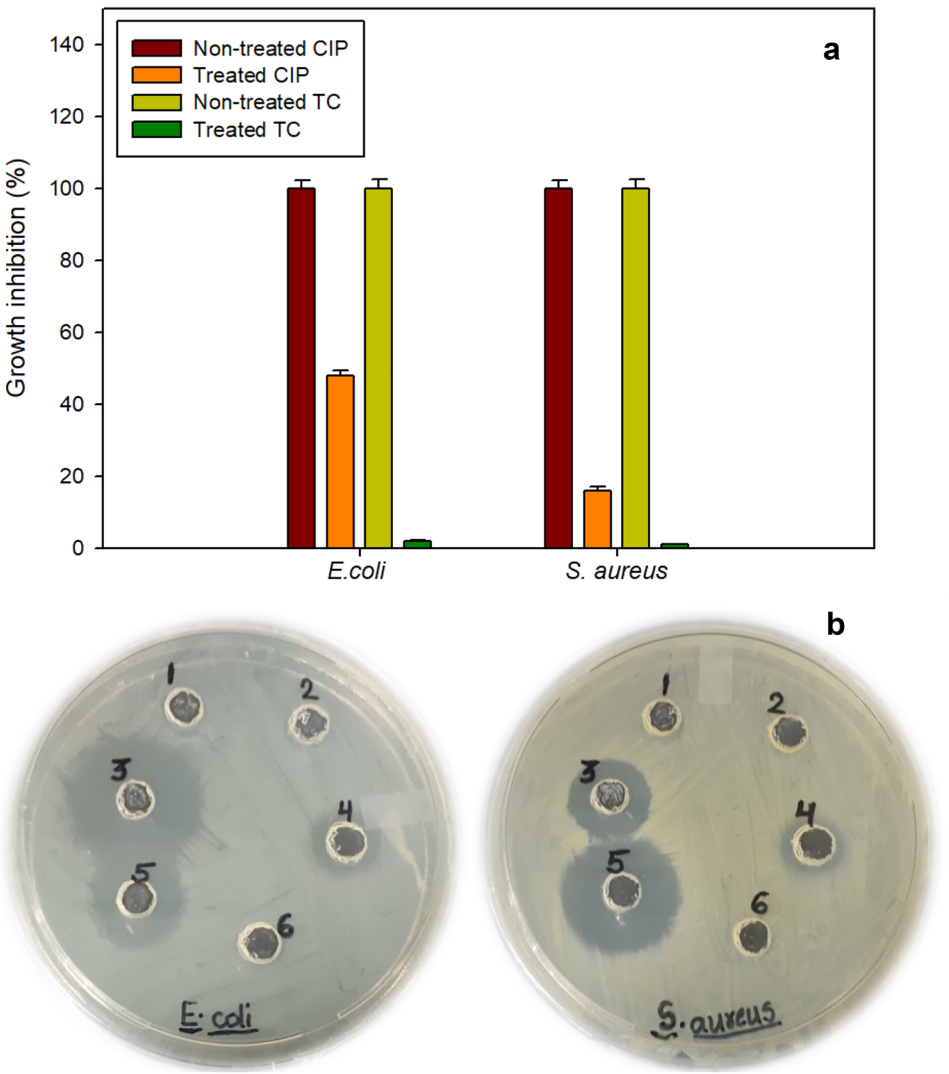

Fig. 6 Antibacterial activity of the products of ciprofloxacin (CIP) and tetracycline (TC) treatment using *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 as reference organisms. (**a**) Growth inhibition percentage in relation to untreated antibiotics, (**b**) well diffusion assays. Solvent controls: **1** DMSO and **2** Milli-Q; non-treated antibiotics: **3** CIP (2.5 mg/L) and **5** TC (100 mg/L); treated antibiotics: **4** CIP and **6** TC

Conclusions

The use of a double promoter system enhanced the production of rSLAC in *P. pastoris* as compared to a single promoter system. The produced rSLAC removed CIP and TC under the optimised experimental conditions. The degradation products of CIP showed reduction of antibacterial activity against *E. coli* and *S. aureus* while complete removal of antibacterial activity was observed in treated TC. Overall, the double promoter system could be a viable approach for improving the production of the rSLAC. The rSLAC can be a good candidate for the removal of the persistent antibiotics, CIP and TC, from contaminated environments.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12223-021-00894-w.

Funding The National Research Foundation (South Africa) provided financial support (Grant 105889 and 112099). Financial support was received from the Council of Scientific and Industrial Research (CSIR) for student (Deepti Yadav) scholarship.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Disclaimer Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard thereto.

Conflict of interest The authors declare no competing interests.

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