

1 *A Streptomyces lividans* SipY deficient strain as a
2 host for protein production: standardization of
3 operational alternatives for model proteins.

4 Marcel·la V. Gabarró¹, Sonia Gullón², Rebeca L. Vicente², Glòria Caminal³, Rafael P. Mellado², Josep
5 López-Santín¹

6
7 ¹Departament d'Enginyeria Química, Biològica i Ambiental. Escola d'Enginyeria.

8 Universitat Autònoma de Barcelona, 08193, Bellaterra (Cerdanyola del Vallès), Catalonia, Spain.

9 ²Departamento de Biotecnología Microbiana. Centro Nacional de Biotecnología (CNB-CSIC), C/ Darwin 3, Campus
10 de la Universidad Autónoma de Madrid, 28049, Cantoblanco, Madrid, Spain.

11 ³Institut de Química Avançada de Catalunya (IQAC-CSIC), C/ Jordi Girona 18-26, 08034, Barcelona, Catalonia,
12 Spain.

13
14
15 **Corresponding author:** Josep López-Santín

16 josep.lopez.@uab.cat

17 Tel.: +345811806

18 FAX: +345812013

19 **Keywords:** *Streptomyces lividans*, heterologous protein production, agarase, SipY mutant strain

20 **Short title:** *Streptomyces* protein production.

21 Post-print of: Vidal Gabarró, M. et al. "A *Streptomyces lividans* SipY deficient strain as a
22 host for protein production: standardization of operational alternatives for model proteins" in Journal of chemical technology and biotechnology (Ed. Wiley), vol. 92, issue 1 (Jan. 2017), p. 217-223. The final version is available at DOI 10.1002/jctb.4933

Abstract

BACKGROUND: Extracellular protein production by Gram-positive bacteria, such as *Streptomyces*, may be complementary to current established protein production processes. The performance of a *Streptomyces lividans* mutant strain, deficient in the major signal peptidase (SipY) is investigated for the production of proteins secreted via the secondary Tat pathway.

RESULTS: The SipY deficient strain has shown advantages over the wild type strain, in terms of extracellular productivity, specific activity and rheological behaviour. Two operational modes: batch and fed-batch have been studied using mannitol as carbon source. The results showed that two successive mannitol additions in fed-batch mode led to improved secretory protein production using *Streptomyces* agarase as a model protein. This production process was also explored for the Tat secretory protein *S. lividans* laccase. The predicted sequence for the pre-laccase coding sequence has been cloned into the mutant strain under the control of the agarase promoter. Batch and fed-batch laccase production, using either mannitol or glucose as carbon source, have been developed and quantified.

CONCLUSIONS: The usefulness of a *Streptomyces lividans* SipY deficient strain as protein producer has been demonstrated. A proposed operation mode with substrate additions has been employed for the optimisation of Tat proteins production, although some adjustments might be necessary depending on the secretory protein.

Introduction

Actinomycetes are ubiquitous soil bacteria containing a high G+C genome¹, and the *Streptomyces* genus belongs to the actinomycetes family and encompasses aerobic mycelia-forming soil bacteria, which are natural producers of many antibiotics and other biologically active molecules². A particularly appealing property of the *Streptomyces* species is its natural ability to produce and secrete extracellular proteins³. This, together with the knowledge acquired over the years on the genetic manipulation of these microorganisms¹ and the accumulated scale-up cultivation experience, have rendered the streptomycetes as a group of interesting bacteria to be exploited in Biotechnology, expressing and secreting extracellular proteins of industrial application⁴⁻⁶. *Streptomyces lividans* is the most utilised species for protein production due to its limited restriction-modification system and relative low endogenous protease

activity⁷. In the last ten years, several proteins have been expressed in this strain with variable active protein concentrations (1-10 mg.L⁻¹ in some cases, up to 300 mg.L⁻¹ in others)^{4,5,8-10}.

Secretory proteins are synthesised by means of a signal peptide at their amino end that helps to trigger the protein to the translocation machinery present at the cell membrane. Type I signal peptidases (Type I SPases) are responsible for cleaving the signal peptide that allows the release of the translocated proteins to the culture broth. Four type I signal peptidases are identified in *Streptomyces lividans*: Sip W, SipX, SipY and Sip Z¹¹. SipY appears to be the major SPase, since the secretome of a SipY defective *S.lividans* strain is severely affected in this strain¹². Despite this handicap, the SipY deficient strain was shown to be very useful for extracellular protein production in *S. lividans*, because the absence of the major signal peptidase was partially compensated by the three remaining active signal peptidases. This permitted the efficient overproduction of extracellular enzymes when their genes were propagated in multicopy as has been revealed for agarase¹³ in the absence of extracellular proteases. The diminished extracellular proteolytic activity should favour the extracellular accumulation of the overproduced protein, potentially improving its stability and the diminished presence of the otherwise naturally-produced extracellular proteins¹² should considerably simplify the downstream processing of the secreted overproduced protein. Bacterial proteins using the secondary Tat (Twin Arginine Translocation) secretory pathway are naturally released to the medium properly folded and fully functional^{14,15}. Therefore, the overproduction of a Tat-secreted protein by an *S. lividans* SipY deficient strain should potentially render a considerable amount of correctly folded, highly stable extracellular product. The practical implementation of protein production processes requires the development of operational procedures to maximise protein productivity. The first objective of this work is to improve process development using a Tat-dependent protein, agarase, as a model to study its overproduction by the SipY mutant strain, with special emphasis on the bioreactor operation mode.

Additionally, the Sip Y deficient strain has been used for the production of *Streptomyces lividans* laccase (Small laccase, SLAC), predicted to be secreted via the Tat pathway. The *Streptomyces coelicolor* laccase was characterized in 2004¹⁶ as a two-domain protein and showed high thermal stability, detergent resistance and an optimum pH in the basic range (around 8). This last fact is remarkable because, although some laccases of fungal¹⁷ and algal origin¹⁸ have been reported to exert the maximum activity at neutral pH, most of the commercially available laccases-generally from fungal origin- have their optimum at acidic pH. Hence, SLAC is a promising enzyme for pollutants' degradation in liquid effluents near

neutral pH like urban wastewater. In addition the *Streptomyces* SLACs could also be useful as a biocatalyst for biotransformations performed in the basic pH range; i.e. in single pot multienzymatic reactions, coupling oxidation and aldol addition¹⁹. In consequence, the possibility of efficient extracellular production of SLAC by the *S. lividans* SipY deficient strain is worth investigating, and the bioreactor operation alternatives developed for agarase were extended to SLAC synthesised in the SipY deficient strain.

Materials and methods

Bacterial strains and plasmids

S. lividans TK21, is a plasmidless derivative of *S. lividans* 66 (John Innes Center Collection, Norwich UK) and was a generous gift from D. A. Hopwood. *S. lividans* TK21Y62 has a deleted *sipY* gene and is deficient in the *S. lividans* major signal peptidase (Palacín et al. 2002). Plasmid pAGAs5 is a pIJ486 derivative carrying the *S. coelicolor* agarase coding gene, *dagA*, and its regulatory region. This plasmid was used to transform *S. lividans*TK21 and *S. lividans* TK21Y62 protoplasts to generate *S. lividans* TK21(pAGAs5) and *S. lividans* TK21Y62(pAGAs5), respectively. Plasmid pFD-PdagA-laccase is a pFD666 derivative carrying the *dagA* promoter and the *S. lividans* laccase coding gene, *slac*, without its regulatory region. The plasmid was used to transform *S. lividans* TK21Y62 protoplasts to generate *S. lividans* TK21Y62(pFD-PdagA-laccase).

Media and culture conditions

Culture Media

Mycelia were routinely stored at -80°C in the presence of glycerol 50% w/v. Pre-inoculum and inoculum for bioreactor cultures were grown in R5 medium¹. Medium for the growth of *S. lividans* TK21(pAGAs5) was supplemented with kanamycin up to a 10µg·mL⁻¹ final concentration in liquid medium and 50µg·mL⁻¹ in solid medium and for *S. lividans* TK21Y62(pAGAs5) also with thiostrepton (10µg·mL⁻¹ final concentration in liquid medium and 50µg·mL⁻¹ in solid medium).

Batch and fed-batch cultures were performed in NMMP medium¹ with 1% mannitol or glucose as carbon sources. This medium contains: 2g·L⁻¹ (NH₄)₂SO₄, 0.6g·L⁻¹ MgSO₄·7H₂O, 5g·L⁻¹ CasAA, 1.8g·L⁻¹ NaH₂PO₄, 2.6g·L⁻¹ K₂HPO₄ and 1.25mL·L⁻¹ of microelements solution (1g·L⁻¹ ZnSO₄·7H₂O, 1g·L⁻¹ FeSO₄·7H₂O, 1g·L⁻¹ MnCl₂·4H₂O and 1g·L⁻¹ CaCl₂ anhydrous). Cultures with *S. lividans* TK21Y62 (

pFD-PdagA-laccase) were supplemented with tryptophan, glycine and CuSO₄ (5mM, 5mM and 5μM final concentration in liquid medium, respectively).

Mycelium stored at -80°C was used to inoculate 3mL of R5 medium with thiostrepton and/or kanamycin as required and set to grow in 50-mL falcon flasks. After 24h of incubation at 30°C and 150 rpm, the cells were transferred to 500mL or 1L baffled flasks containing 50mL or 250mL of the same medium. These cultures were grown for 24h at 30°C and 150 rpm and the corresponding biomass harvested by centrifugation; biomass at an initial concentration of 0.1g·L⁻¹ of wet weight was used to inoculate a 3L bioreactor or a 7 L bioreactor.

Bioreactor batch cultures

One and a half litres of *S. lividans* TK21(pAGAs5), *S. lividans* TK21Y62 (pAGAs5) and *S. lividans* TK21Y62(pFD-PdagA-laccase) batch cultures were set to grow in a 3-litre Applikon Bioreactor ez-Control. The culture conditions were controlled and monitored at the following set points: temperature 30°C; pH 7.0±0.05 with the addition of 2M of NaOH and 2M of HCl; pO₂ at 50% saturation by controlling the stirring speed between 200rpm and 800rpm and using an aeration of 1.5vvm of air enriched with O₂ when necessary.

Bioreactor fed-batch cultures

S. lividans TK21Y62(pAGAs5) and *S. lividans* TK21Y62(pFD-PdagA-laccase) fed-batch cultures were performed in a 7-L Applikon Bioreactor working at an initial volume of 3.5L. Culture set points were same as for batch cultures. As described before, NMMP medium was used for the initial batch cultivation with supplementations when it was necessary. Pulse additions of carbon source (mannitol (20% w/v) or glucose (20% w/v)) were added to re-establish 10g·L⁻¹ of carbon source at different times as indicated in the Results section. In some experiments pulses of casamino acids (20% w/v) increasing their concentration by 2.5g·L⁻¹ were also performed.

Analytical methods

Biomass concentration

Biomass concentration was measured in 3mL samples by determining the dry weight of cells retained on pre-weighed glass microfiber filters (WhatmanTM, GE Healthcare), which were subsequently washed with two volumes of distilled water and dried at 105°C to constant weight. Measurements were performed in triplicate.

Mannitol and glucose concentration

Mannitol and glucose concentrations were determined by High Pressure Liquid Chromatography (HPLC) with an Ultimate 3000 liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) using an ICSep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA) with an IR detector (Waters 2410). The mobile phase was 8 mM sulphuric acid and the injection volume was 10 μ L.

Extracellular protein concentration

Total extracellular protein was assessed using a Coomassie® Protein Assay Reagent Kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA)²⁰ in microtiter plates using a MultiSkan™ FC microplate reader (Fisher Scientific™). Bovine serum albumin (BSA) was employed as standard protein.

Agarase activity assay

Agarase activity was determined directly from the supernatant via a modification of the previously described method²¹, by quantifying the reducing sugars released as a result of agarase hydrolysis. The volume of the assay was one millilitre: 850 μ L of agarose (0.1% w/v final concentration), 100 μ L of imidazole-HCl (500mM, pH 6.5), 10-50 μ L of unknown sample and milliQ water up to 1mL. One unit of enzyme activity is the amount of enzyme producing an increase of 0.001 units of absorbance at 450nm per minute at 37°C. The time of incubation was 5 minutes and the assay was linear between 0.1 and 0.6 units of absorbance.

Laccase activity assay

Laccase activity was determined directly from the supernatant quantifying 2,6-dimethylphenol (DMP) turnover, monitored at 468nm ($\epsilon = 14.800\text{M}^{-1}\text{cm}^{-1}$ for the dimeric product^{16,22}). All UV-visible measurements were obtained using a UV-VIS Cary (Varian) spectrophotometer. The assay mixture consisted of 750 μ L of Tris-HCl (100mM pH 8.0), 50 μ L of DMP (40mM) and 200 μ L of sample to be assayed. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute.

Protease activity assay

Proteolytic activity in *S. lividans* cultures was assayed by using the QuantiCleave protease assay kit (Pierce). This assay is based on the use of succinylated casein in conjunction with trinitrobenzenesulfonic acid (TNBSA)^{23,24}. Proteases will cleave peptide bonds in the succinylated casein and expose primary amines (predominantly α -amines). Thereafter, TNBSA will react with the primary amines to generate an orange-yellow colour. The colour change was quantified at 450nm by using a microplate reader. The

assay was performed at 25°C for 20 min at pH 8.5 (borate buffer, 50mM). The increase in the absorbance measures the protease activity.

Western blot analysis

Proteins were fractionated by SDS-PAGE in 12 % (w/v) acrylamide gel²⁵ and transferred onto immobilonpolyvinylidenedifluoride membranes (Milipore), as described²⁶. The transferred material was incubated with rabbit polyclonal antibodies raised against *S. coelicolor* agarase (DagA; a gift from V. Parro) followed by incubation with HRP-conjugated protein A (Invitrogen Laboratories) diluted 1:10,000 in PBS containing 5 % (w/v) skimmed milk for 40 min at room temperature²⁷. Peptides reacting with the antibodies were revealed using the ECL enhanced chemiluminescence system from Amersham after one min incubation and different exposures to X-ray film ranging from 20 s to 3 min.

Quantity One (Bio-rad) was used to quantify the band observed after the reaction with the antibodies against DagA.

Results

Batch agarase production

The agarase production by the SipY deficient strain, *S. lividans* TK21Y62(pAGAs5)¹³, was studied in comparison to *S.lividans* TK21(pAGAs5) (from now the wild type strain). Bacterial cell cultures were performed in a 3L bioreactor using either mannitol or glucose as carbon source. The average results concerning biomass growth and agarase production in mannitol for both strains are shown in Table 1. Biomass and total extracellular protein concentration were higher for the wild type than for the SipY deficient strain, while total extracellular agarase and specific agarase activities per gram of biomass and per mg of protein were higher for the deficient strain; this was expected since the accumulation of extracellular proteins is severely diminished in the SipY deficient strain compared to the wild type^{12,13}. Cultures grown in the presence of glucose as carbon source (data not shown) produced 30% less agarase than those grown in the presence of mannitol. The time course of a representative mutant strain culture in the presence of mannitol as carbon source is shown in Figure 1. Biomass growth starts before mannitol consumption, presumably using casamino acids as a carbon source²⁸, with a specific growth rate of $\mu=0.12\text{ h}^{-1}$ at the exponential phase. During extracellular agarase accumulation the protease levels in the medium wer lower than the detection limit.

1 Additionally, the mutant exhibited better rheological properties compared to the wild type. The SipY
2 deficient strain grows in a disperse suspension considerably diminishing cellular adhesion to the reactor
3 wall, a very significant drawback of the wild type strain which grows in clump aggregates. This
4 remarkable disperse growth characteristic may simplify the standardization and scale up of production
5 due to its better rheological behaviour.

6 All the above results suggest that *S. lividans* TK21Y62 can provide advantages for the overproduction of
7 Tat secretory proteins. Further improvement of this production was attempted by extending the bioreactor
8 operation to fed-batch mode.

9 **Fed-batch agarase production**

10 Fed-batch operational mode was assayed as a method to simultaneously increase agarase concentration
11 and productivity (expressed as $\text{U.L}^{-1}.\text{h}^{-1}$). The strategy consisted of mannitol pulse addition, recovering its
12 initial concentration in the culture medium. All the other nutrients were calculated to be in excess of the
13 necessary amount to support the biomass concentration achieved in the process. Pulse addition was
14 performed avoiding total mannitol depletion. As can be seen in Figure 1, when mannitol was totally
15 depleted, biomass concentration decreased due to cell lysis and a total extracellular protein increment was
16 detected in the supernatant, without increasing the agarase activity.

17 Figure 2 shows the time course of one of the described fed-batch cultures. A first mannitol addition was
18 performed at 46h and a second one at 58h, re-establishing the initial mannitol concentration. Biomass
19 growth increased throughout the mannitol addition process. The first mannitol addition significantly
20 increased agarase production (almost twofold), and the second one produced only a slight increase in
21 activity. Further mannitol additions did not exert any positive effect on agarase production (data not
22 shown). Casamino acids were added as indicated in Materials and Methods, yet no significant effect on
23 agarase production was obtained.

24 The relative levels of agarase extracellular activity correlated with the extracellular presence of agarase as
25 estimated by Western blot analysis of fed-batch samples. The intensity of the bands (data not shown)
26 increased until 56h, and remained almost constant henceforth until the end of growth. As a control, a
27 parallel western blot assay was carried out with a non-productive strain, and as expected no reaction
28 signal with anti-Dag was obtained.

Two additional complementary fed-batch cultures were performed: a first one adding casamino acids by pulses instead of mannitol; and a second one doubling the initial casamino acid concentration and with the two mannitol pulses. Both cultures were less productive (data not shown), than the fed-batch culture presented in Figure 2, confirming that the presence of extra casamino acids did not lead to an increase in agarase production.

Batch growth of laccase-producer strains

As a further insight into the possibility of using the SipY deficient strain to improve Tat protein production, the putative Tat protein laccase was used to perform the experiments. The laccase coding sequence and its regulatory region was PCR-amplified from the *S. lividans* TK21 genome and cloned into the multicopy plasmid pFD666 and subsequently transformed into the *S. lividans* TK21Y62 protoplasts showing no detectable laccase activity during growth in batch cultures. The agarase promoter (*PdagA*) employed in the previous section has been described as a strong promoter to obtain efficient protein secretion using *Streptomyces lividans* as a host²⁹. The laccase coding region was amplified from *S. lividans* and cloned into a multicopy plasmid under the control of the agarase promoter (*PdagA*), generating the plasmid pFD-*PdagA*-laccase, and the plasmid was propagated in the SipY defective strain. The bacterium was cultured in a 5L batch bioreactor using an NMMP modified medium as indicated in the Materials and Methods section with 10g·L⁻¹ of either mannitol or glucose as carbon sources. In both conditions sustained growth and laccase synthesis and secretion were obtained. Batch growth on mannitol is depicted in Figure 3A.

Growth was maintained until the total depletion of the carbon source occurred, with a specific growth rate of 0.09 h⁻¹ at the exponential phase. The behaviour of the mutant strain was as expected, producing a low amount of secretory proteins due to the major signal peptidase mutation. Laccase activity accumulated extracellularly until a maximum of 5.8 U.L⁻¹, following a temporal secretion pattern compatible with a protein secreted via the Tat pathway³⁰. As in the case of agarase, extracellular protease levels were not detectable and a decrease in biomass concentration and laccase activity took place after total mannitol consumption, which was attributed to cell lysis and possible laccase degradation by intracellular released proteases. A similar result was obtained in glucose batch cultures (Fig 3B), with a higher specific growth rate, (0.15 h⁻¹), reaching a maximum biomass concentration of 7.5 g.L⁻¹. The biomass concentration also decreased at the end of culture, probably due to cell lysis. Extracellular laccase accumulation followed the

expected pattern although the secreted amount was lower, 3.1 U.L⁻¹, and no activity decay parallel to the cell lysis was observed.

In summary, maximum values of laccase production, under comparable conditions, are presented in Table 2. As can be seen, laccase activity is considerably higher for mannitol than for glucose, in terms of total production, specific production per gram of biomass and specific production per mg of extracellular protein.

Fed-batch cultures for laccase production

Agarase production by the SipY deficient strain increased with one single addition of mannitol and further additions of carbon or nitrogen sources did not improve the process (Fig 2). Therefore, this operational strategy has been applied for laccase production with mannitol or glucose fed-batch additions, restoring the initial 10 g.L⁻¹ concentration of the carbon sources. The time profiles for both cultures are shown in Figure 4. As can be seen, mannitol addition led to a moderate increase in laccase extracellular activity while glucose addition exerted a drastic effect by almost doubling the enzyme activity.

In the mannitol fed-batch culture (Fig. 4A), maximum biomass concentration and laccase activity were obtained after mannitol addition at 82 h. Conversely, maximum biomass concentration and laccase activity for the glucose fed-batch (Fig 4B) cultures with two glucose additions were obtained earlier, at 56 h. In both cases, the extracellular protein concentration increase was concomitant to a biomass decrease, which also coincided with a decrease in laccase activity, a possible consequence of laccase degradation by the intracellular proteases released on account of the bacterial cell lysis.

Discussion

The use of the SipY deficient strain as a host for agarase overproduction affords some remarkable advantages compared to the wild type strain: higher agarase specific activity, more reproducibility of cultures and a non-sporulated phenotype. Extracellular protein concentration is lower for the SipY deficient strain, with almost undetectable protease activity, maintaining overexpression and secretion of agarase.

Mannitol was a better carbon source for the agarase production by the SipY deficient strain grown in batch cultures; the use of glucose as carbon source revealed a lower agarase production, as previously described²¹.

The fact that bacterial growth starts before mannitol consumption, presumably using casamino acids present in the medium as carbon source (Figure 1), was considered in the design of fed-batch experiments to increase biomass concentration. Successive additions of both, mannitol and casamino acids was assayed. Nevertheless, only the strategy with two mannitol additions successfully increased biomass concentration, leading to higher agarase activity. Subsequent mannitol additions were ineffective, limiting the improvement obtained in the fed-batch operation to the one depicted in Figure 2. Casamino acid additions did not increase cell growth neither agarase production. In the absence of any other nutrient limitation, this may be explained from the characteristics of the expression system. The *S. coelicolor* *dagA* gene was cloned in a high copy number plasmid¹³ and expressed under the control of its own promoter. As expression is constitutive, it cannot be decoupled from growth, competing for nutrient resources, and, as a result, when the cellular growth stops, gene expression stops as well, which results in no increase of agarase synthesis.

A comparison of the two operation modes: batch, and fed-batch was carried out for agarase production (Table 3). Higher productivity was obtained using the fed-batch process, which was feasible for practical application to produce the model protein agarase in a bench-scale bioreactor. The fed-batch operation with two mannitol additions resulted in the production of a more concentrated agarase, facilitating a much easier downstream processing of the extracellular enzyme. As expected, the estimated specific agarase activity per mg of protein in batch and fed-batch operation is nearly the same (Table 3), since in both cases agarase itself was the main contributor to the extracellular protein concentration.

Concerning laccase, a comparison of fed batch culture production in the presence of mannitol or glucose as carbon source is presented in Table 4, comprising the data obtained at periods reflecting the effect of each pulse (see Figure 5). A similar production of laccase (5.35 U.L⁻¹) is obtained after 55 hours' growth, corresponding to either a batch mannitol culture or to two additions in fed-batch glucose culture. Consequently, the operation in fed-batch mode with mannitol or glucose additions led to similar productivities yet with three fold more substrate consumption in the case of the glucose. Nevertheless, for process optimization, it must be considered that mannitol price per Kg is more than six fold higher than glucose and the proposed fed-batch operation with glucose as carbon source seems to afford more economic incentives.

In conclusion, the above results confirm the potentiality of the SipY defective strain as a producer of proteins secreted via the Tat pathway, constituting the guidelines and a primary approach for the expression of such proteins.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (project number CTQ2011-28398-CO2-01), the Spanish Ministry of the Environment and Rural and Marine affairs (Grant No. EGO22008) and Grant PIE201220E003 from the CSIC.

The authors from UAB are members of the Research group 2014SGR452 and of the Biochemical Engineering Unit of the Reference Network in Biotechnology (XRB), Generalitat de Catalunya.

References

1. Kieser T et al. *Practical Streptomyces Genetics*. The John Innes Foundation. Norwich, England (2000).
2. Chater KF. Streptomyces inside-out: a new perspective on the bacteria that provide us with antibiotics. *Philos Trans R Soc Lond B Biol Sci* 361:761–768. doi: 10.1098/rstb.2005.1758 (2006).
3. Gilbert M, Morosoli R, Shareck F, Kluepfel D. Production and secretion of proteins by streptomycetes. *Critical reviews in Biotechnology* 15:13–39 (1995).
4. Anné J, Maldonado B, Van Impe J, Van Mellaert L, Bernaerts K. Recombinant protein production and streptomycetes. *J Biotechnol* 158:159–167. doi: 10.1016/j.jbiotec.2011.06.028 (2012).
5. Noda S, Ito Y, Shimizu N, Tanaka T, Ogino C, Kondo. A Over-production of various secretory-form proteins in *Streptomyces lividans*. *Protein Expr Purif* 73:198–202. doi: 10.1016/j.pep.2010.05.011(2010).
6. Sianidis G, Pozidis C, Becker F, Vrancken K, Sjoeholm C, Karamanou S, Takamiya-Wik M, Van Mellaert L, Schaefer T, Anné J, Economou A. Functional large-scale production of a novel Jonesia sp. xyloglucanase by heterologous secretion from *Streptomyces lividans*. *J Biotechnol* 121:498–507. doi: 10.1016/j.jbiotec.2005.08.002 (2006).
7. Nakashima N, Mitani Y, Tamura T. Actinomycetes as host cells for production of recombinant proteins. *Microb Cell Fact* 4:7. doi: 10.1186/1475-2859-4-7 (2005).

8. Li JX, Zhao LM, Wu RJ, Zheng ZJ, Zhang RJ. High-level overproduction of Thermobifida enzyme in *Streptomyces lividans* using a novel expression vector. *Int J Mol Sci* 14:18629–18639. doi: 10.3390/ijms140918629 (2013).
9. Noda S, Miyazaki T, Miyoshi T, Miyake M, Okai N, Tanaka T, Ogino C, Kondo. A Cinnamic acid production using *Streptomyces lividans* expressing phenylalanine ammonia lyase. *J Ind Microbiol Biotechnol* 38:643–648. doi: 10.1007/s10295-011-0955-2 (2011).
10. Noda S, Miyazaki T, Tanaka T, Ogino C, Kondo. A Production of *Streptoverticillium cinnamoneum* transglutaminase and cinnamic acid by recombinant *Streptomyces lividans* cultured on biomass-derived carbon sources. *Bioresour Technol* 104:648–651. doi: 10.1016/j.biortech.2011.10.045 (2012).
11. Parro V, Schacht S, Anné J, Mellado RP. Four genes encoding different type I signal peptidases are organized in a cluster in *Streptomyces lividans* TK21. *Microbiology* 145:2255–2263 (1999).
12. Escutia MR, Val G, Palacín A, Geukens N, Anné J, Mellado RP Compensatory effect of the minor *Streptomyces lividans* type i signal peptidases on the SipY major signal peptidase deficiency as determined by extracellular proteome analysis. *Proteomics* 6:4137–4146. doi: 10.1002/pmic.200500927 (2006).
13. Palacín A, Parro V, Geukens N, Anné J, Mellado RP SipY is the *Streptomyces lividans* type I signal peptidase exerting a major effect on protein secretion. *J Bacteriol* 184:4875–4880. doi: 10.1128/JB.184.17.4875-4880.2002 (2002).
14. Berks BC, Palmer T, Sargent F. Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. *Curr Opin Microbiol* 8:174–181. doi: 10.1016/j.mib.2005.02.010 (2005).
15. Gullón S, Vicente RL, Mellado RP. A Novel Two-Component System Involved in Secretion Stress Response in *Streptomyces lividans*. *PLoS ONE*. doi: 10.1371/journal.pone.0048987 (2012).
16. Machczynski MC, Vijgenboom E, Samyn B, Canters GW Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. *Protein Sci* 13:2388–2397. doi: 10.1110/ps.04759104 (2004).

17. Berka RM, Schneider P, Golightly EJ, Brown SH, Madden M, Brown M, Halkier T, Mondorf K, Xu F. Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. *Appl Environ Microbiol* 63:3151–3157 (1997).
18. Otto B, Schlosser D. First laccase in green algae: purification and characterization of an extracellular phenol oxidase from *Tetracystis aeria*. *Planta* 240:1225–1236. doi: 10.1007/s00425-014-2144-9 (2014).
19. Pešić M, López C, López-Santín J, Alvaro G. From amino alcohol to aminopolyol: one-pot multienzyme oxidation and aldol addition. *Appl Microbiol Biotechnol* 97:7173–83. doi: 10.1007/s00253-013-5011-x (2013).
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248–254 (1976).
21. Parro V, Mellado RP. Effect of glucose on agarase overproduction by *Streptomyces*. *Gene* 145:49–55 (1994).
22. Solano F, Lucas-Elío P, López-Serrano D, Fernández E, Sanchez-Amat A. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. *FEMS Microbiol Lett* 204:175–181. doi: 10.1016/S0378-1097(01)00400-1 (2001).
23. Bubnis WA, Ofner III CM. The determination of ϵ -amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. *Analytical Biochemistry* 207:129–33 (1992).
24. Gullón S, Marín S, Mellado RP. Overproduction of a Model Sec- and Tat-Dependent Secretory Protein Elicits Different Cellular Responses in *Streptomyces lividans*. *PLoS ONE* 10(7):E0133645. doi: 10.1371/journal.pone.0133645 (2015).
25. Laemmli UK. Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature* 227:680–688 (1970).
26. Timmons TM, Dunbar BS. Protein blotting and immunodetection. *Methods in Enzymology* 182:679–688 (1990).

- 1 27. Rabindran SK, Haroun RI, Clos J, Wisniewski J, Wu C. Regulation of heat shock factor trimer
2 formation: role of a conserved leucine zipper. *Science* 259:230–234. doi: 10.1126/science.8421783
3 (1993).
- 4 28. D’Huys PJ, Lule I, Van Hove S, Vercammen D, Wouters C, Bernaerts K, Anné J, Van Impe JFM.
5 Amino acid uptake profiling of wild type and recombinant *Streptomyces lividans* TK24 batch
6 fermentations. *J Biotechnol* 152:132–143. doi: 10.1016/j.jbiotec.2010.08.011 (2011).
- 7 29. Isiegas C, Parro V, Mellado RP. *Streptomyces lividans* as a host for the production and secretion of
8 *Escherichia coli* TEM β -lactamase. *Lett Appl Microbiol* 28:321–326. doi: 10.1046/j.1365-
9 2672.1999.00519.x (1999).
- 10 30. Hatakeyama T, Kohzaki H, Yamasaki N. A microassay for proteases using succinylcasein as a
11 substrate. *Analytical Biochemistry* 204:181–184 (1992).

FIGURE CAPTIONS

Figure 1. Time course of SipY-deficient strain batch growth on mannitol. (○)Biomass concentration ($\text{g}\cdot\text{L}^{-1}$);(◇) mannitol concentration ($\text{g}\cdot\text{L}^{-1}$); (Δ) extracellular protein concentration ($\text{mg}\cdot\text{L}^{-1}$); (▲) extracellular agarase activity ($\text{kU}\cdot\text{L}^{-1}$).

Figure 2. Time-course of fed-batch fermentation with SipY deficient strain with mannitol pulse addition (mannitol added at 46h and 58h). A) (○)Biomass concentration ($\text{g}\cdot\text{L}^{-1}$);(◇) mannitol concentration ($\text{g}\cdot\text{L}^{-1}$) B) (Δ) extracellular protein concentration ($\text{mg}\cdot\text{L}^{-1}$); (▲) extracellular agarase activity ($\text{kU}\cdot\text{L}^{-1}$).

Figure 3. Time course of *S. lividans* TK21Y62(pFD-PdagA-laccase) batch growth on A) mannitol and B) glucose. (○)Biomass concentration ($\text{g}\cdot\text{L}^{-1}$);(◆) mannitol / glucose concentration ($\text{g}\cdot\text{L}^{-1}$); (Δ) extracellular protein concentration ($\text{mg}\cdot\text{L}^{-1}$); (●) extracellular laccase activity ($\text{U}\cdot\text{L}^{-1}$).

Figure 4. Time course of *S. lividans* TK21Y62 (pFD-PdagA-laccase) fed-batch growth on A) mannitol and B) glucose. (○)Biomass concentration ($\text{g}\cdot\text{L}^{-1}$);(◆) mannitol / glucose concentration ($\text{g}\cdot\text{L}^{-1}$); (Δ) extracellular protein concentration ($\text{mg}\cdot\text{L}^{-1}$); (●) extracellular laccase activity ($\text{U}\cdot\text{L}^{-1}$).