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HETEROLOGOUS PROTEIN EXPRESSION OF THE BOVINE *LIPOPROTEIN LIPASE* IN *Aspergillus nidulans* FOR MILK PRESERVATION

TIPO TESI: sperimentale

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To Resilience.

INDEX

Abstract

The *Lipoprotein Lipase* (LPL) is a highly conserved protein with a key role in triglycerides (TGs) metabolism. The enzyme catalyses the hydrolysis of TGs into monoacylglycerols and fatty acids (FFA) in the bloodstream and inside mammocytes. In humans, defective LPL causes a rare and potentially fatal disease named Familial Chylomicronemia Syndrome (FCS). LPL is also found in cow's milk, where it could be responsible for rancidity, acting on fat globules. Bovine LPL (bLPL) is usually purified from cow's milk albeit with very low yields. In order to deeply investigate the role and potential biotechnological applications of bLPL, it is essential to establish a system for the recombinant production of this protein in its active form. To this purpose a new system based on *Aspergillus nidulans* is here presented. *A. nidulans* is a filamentous fungus, of the *phylum Ascomycota*. *Aspergillus* genome has been sequenced and the availability of data allows gene manipulation of this microbial genus for heterologous protein expression. Moreover, *A. nidulans* is cheap to grow, with the possibility of utilizing several selection markers and inducible gene promoters. In this work AlcA plasmid is used as vector for the recombinant expression of bLPL, under the control of the ethanol-inducible Alcohol dehydrogenase promoter. Obtained *Aspergillus* recombinant strains were analysed for the presence of bLPL sequence within the genome and assayed for their viability after induction of the protein expression. Selected transformants were then tested for their capability to express bLPL through proteomic analyses. Lastly, affinity chromatography was applied to the produced bLPL to establish the purification procedure.

Keywords: *Lipoprotein Lipase*- *Aspergillus nidulans*- recombinant expression- protein purification- milk rancidity

1. INTRODUCTION

Mankind has been using enzyme preparations in food processing for centuries. For instance, one of the most known formulations is curdled milk from the stomach of an unweaned calf, containing the protease chymosin, also popularly known as rennin, and used in curdling milk for cheese.

Nowadays the industrial production of pure enzymes relies on the recombinant expression and purification from microorganisms. The production of recombinant enzymes from microbes, yeast or fungi is an advantageous strategy due to the achievement of high products yields (proteins, organic acids, hormones, etc.), the tight process control and cost-effectiveness.

Lipases, in particular, are a family of enzymes catalyzing hydrolysis of long chain triglycerides (TGs), widely exploited in food processing to break down fats in cheese making, as emulsifiers in baking and egg yolk preparations, in infant formulas, oil production and as cocoa butter substitutes (Guerrand, 2017; Geritz *et al*, 2014).

Examples of recombinant lipases are *Candida Antarctica* lipase B, used in the industry of flavours and emulsifiers, and the *Rhizopus chinensis* lipase (RCL), used in bakery to improve volume and rheology of bread loaves (Borrelli and Trono, 2015).

The present thesis work focused on the heterologous expression of the bovine *lipoprotein lipase* (EC 3.1.1.34, bLPL) in *Aspergillus nidulans* in order to set a new cell factory able to produce an active bLPL. Once obtained, recombinant bLPL will be utilized for future determination of the protein structure in order to improve the knowledge about its functions in the milk and detect possible strategies to prevent bLPL involvement in milk rancidity.

Therefore, an overview of the main known characteristics of the *lipoprotein lipase* and of the filamentous fungus *A. nidulans* will follow.

1.1 Origin of bovine *Lipoprotein lipase*

The bovine serum *lipoprotein lipase* is also responsible for FFA uptake into cells of the mammary glands (Patton, 1975; Emery, 1973). Mammocytes, on the other hand, produce their own bLPL which is also involved in milk triglycerides re-synthesis using FFA as substrates. Re-synthesised TGs partly coalesce into fat globules to be secreted in milk and are partly re-hydrolysed by the bLPL to be re-secreted into the blood stream in a recycling cycle. No biological function for the bLPL in milk has been elucidated until now (Deeth, 2006). The amount of bLPL produced in the mammary glands depends on breed, cow age, diet and hormonal inputs (estrogen, prolactin, estradiol) (Emery, 1973; Chandan and Shahani, 1964; Hohe *et al.*, 1984; Chen *et al.*, 2003).

bLPL has a molecular weight (MW) between 56 and 66kDa (Jensen and Pitas, 1976), probably due to its glycosylation levels. The optimum pH is 8.7 for enzymatic activity. It is inhibited by 1M of NaCl and enhanced by NH_4^+, Ca^{2+} and BSA, which works also as a stabilising factor (Jensen, 1976; Hayashi, 1986). ApoC2 (apolipoprotein C2) can also increase bovine LPL activity (Ganesan *et al.*, 1971; Have *et al.*, 1973; Hayashi *et al.*, 1986).

The bovine LPL is thought to enter the milk through the lactation process directly and indirectly (Emery, 1973; Jensen and Pitas, 1976; Deeth, 2006; Patton, 1975). In brief, during lactation, the cells of the mammary gland receive nutrients from the arterial blood. On the other hand, metabolites produced to form the milk “tissue” are secreted in two ways: proteins and casein micelles that contain also the bLPL follow the Endoplasmatic Reticulum (ER)-Golgi pathway and eventually are secreted when Golgi vesicles release their content into the mammary gland lumen; fat droplets, on the contrary, are embedded by the mammary gland membrane facing the lumen and secreted as fat globules (Figure 1). This mechanism involves the continuous disruption and regeneration of all membranes (ER, Golgi, cell membranes) and also the possibility of material leaking, found sometimes in the milk as cell debris. This mechanism is well described in Patton (1975).

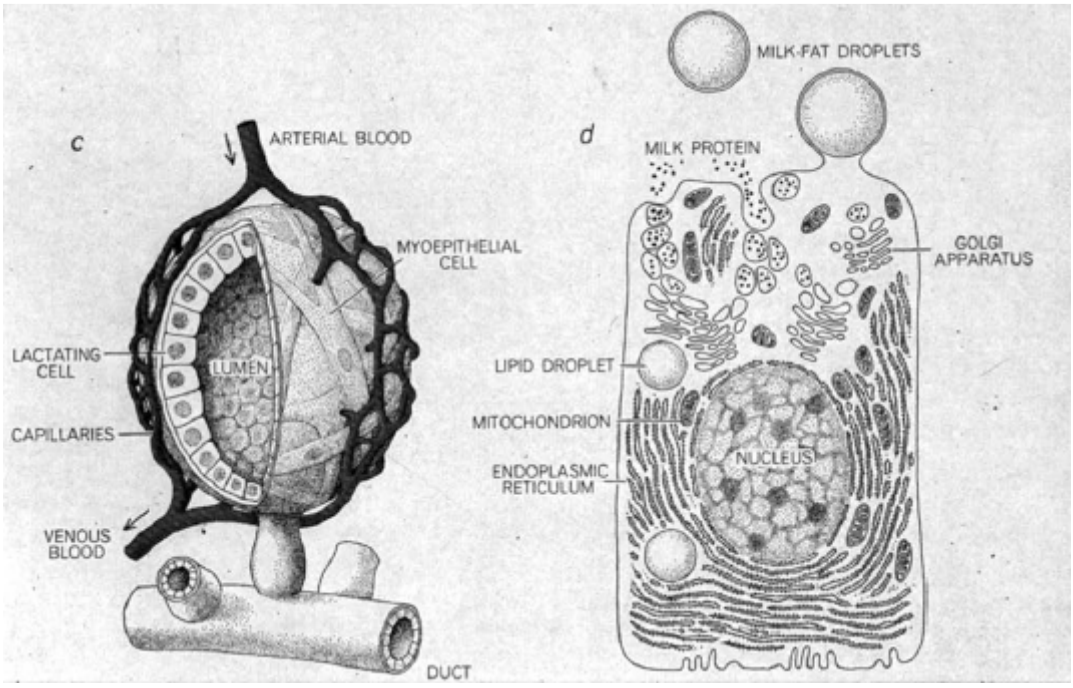


Figure 1 Lactation mechanism in bovines. c) structure of a single alveolus showing the arrangement of the mammary gland cells and (hollow) lumen area into which milk is secreted, (d) a scheme of the mammary cell during lactation illustrating the mechanisms of protein and fats secretion and showing the principal membrane systems of the cell, i.e., ER, mitochondrion, Golgi apparatus and plasma membrane (cell envelope). (From *Milk,* by S. Patton, 1969)

bLPL reaches milk embedded in casein micelles and also though leakage (Emery, 1973; Jensen and Pitas, 1976; Deeth, 2006; Patton, 1975).

Milk contains an average of 1-2mg of LPL per liter, mainly bound to caseins (Olivecrona, 1980). Its location in the milk "tissue" has been explored and it depends on milk handling: generally speaking, it is mainly present in casein micelles and in smaller amounts in milk fluff (cellular debris that forms around 12h after lactation), in milk serum as free form and traces can be found bound to fat globules (Hohe *et al.*, 1984).

bLPL is relatively unstable and it stays inactive after traditional thermal treatments like High Temperature Short Time pasteurization (Farkye *et al.*, 1995) but when the milk is raw, it is the main if not the only enzyme responsible for milk rancidity

(Deeth, 2006; Deeth and Fitz-Gerald, 2006; Sundheim and Bengtsson-Olivecrona, 1984; Ray*et al.*, 2013; Chen 2003; Bauchman *et al.*,1987).

As long as milk fat globules remain intact bLPL never gets in touch with TGs. Following sudden freezing or mechanical shock, fat globules can disrupt, and triglycerides are free to react with enzymes causing off-flavour and off-taste due to the release of short and medium chain FFA. In some cheeses, though, it can be a desirable effect, like in blue cheeses.

1.2 Phylogenetic relationship of *Lipoprotein lipase*

LPL protein is a highly conserved lipase amongst mammals (Figure 2) and also between mammals and birds (Hide *et al*, 1992; Mead *et al.*, 2002).

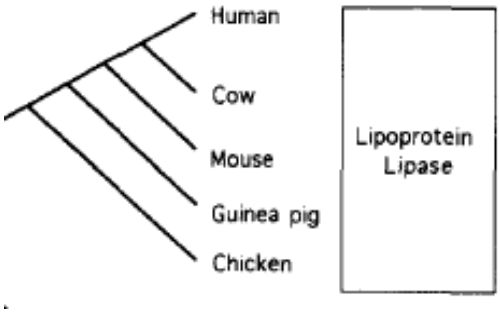


Figure 2 Phylogenetic relationship of the LPL family (Hide *et al.*, 1992)

As shown in Figure 3, the human and bovine LPL amino acid sequences show the lowest rate of residues substitution. As one can expect, the highest evolutionary distance is with the chicken, which does not belong to the Mammalian class (Figures 2 and 4). By performing a simple alignment of the protein sequences, we can get identities as follows: human-cow have 92% of identities with most of the differences occurring at the N- and C- terminal ends (Figure 3). Human and rabbit (not included in Hide's study) have 93% of identities; human and pig 91%, human and chicken 76% as shown in Figure 4.

Figure 3 Alignment of human and bovine LPL (BLAST).Query and Subject correspond to the human and bovine LPL amino acid sequences respectively.

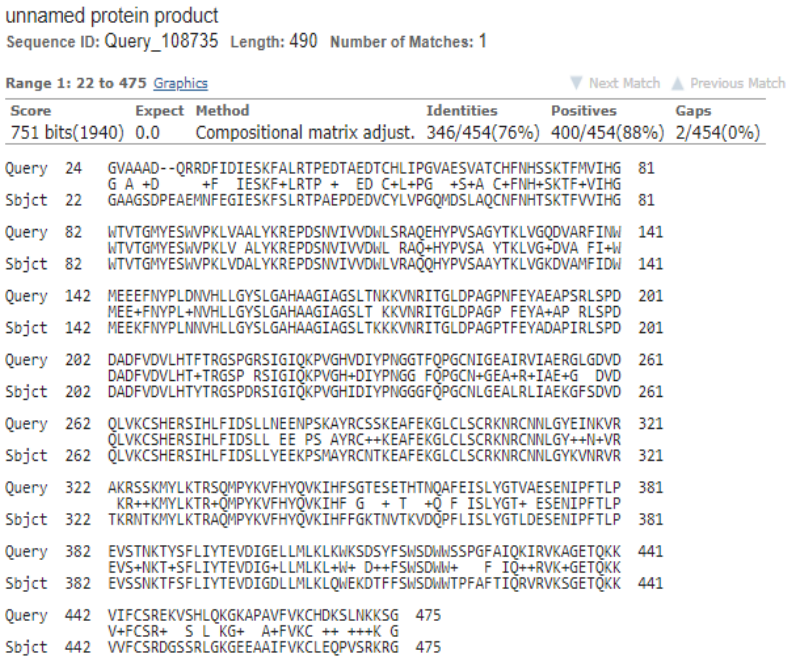


Figure 4 Alignment of human and chicken LPL sequences (BLAST). Query and Subject correspond to the human and chicken LPL amino acid sequences respectively.

1.3 Structure and functions

LPL is a glycoprotein (Olivecrona and Bengtsson, 1983; Olivecrona *et al.*, 2003) and according to the Enzyme Commission (EC) number it is a hydrolase (EC 3) that acts on ester bonds (EC 3.1), and it is characterized as a carboxylic ester hydrolase (EC 3.1.1) of its own (EC 3.1.1.34) (de Andrade Jr, 2018). LPL is synthesized in various tissues- mostly in the heart, skeletal muscle, adipose tissue, nervous system, liver, mammary gland, kidney, adrenals, pancreatic islet cells, lungs and spleen- where it exerts different functions relating to lipid metabolism and transport (He *et al.*, 2018). Moreover, it is synthesized and afterwards processed within the ER, where it is glycosylated (Mead *et al.*, 2002) and properly folded thanks to a specific chaperone like the Lipase Maturation Factor 1 (LMF1) and the adaptor Sel1L.

The human LPL gene is mapped on the short arm of chromosome 8 and it encodes for a 448 amino acids-long protein, comprising 10 exons and 9 introns (Wang and Eckel, 2009; Mead *et al.*, 2002). The first exon encodes also for the whole signal peptide (Kirchgessner *et al.*, 1989). The protein is made up of two distinct regions, one is the larger amino terminal domain (residues 1-312), the other is the carboxy-terminal domain (319-448), connected together by a flexible peptide. The N-terminal domain contains the active site triad (Ser-132, Asp-156, His-241) and a 22 amino acids-long lid covering the site, making it responsible for the catalysis (Todd *et al.*, 2007; He *et al.*, 2018; Dugi *et al.*, 1992). The active form is a homodimer of two non-covalently bound subunits with a head-to-toe configuration (Bengtsson-Olivecrona *et al*, 1986; Otarod and Goldberg, 2014). Thanks to restriction enzymes trials, we know that the two domains are identical in size for both the human and the bovine homologs (Bengtsson-Olivecrona *et al.*, 1986). It has been demonstrated that its cofactor ApoC2 is essential for the enzyme to successfully bind lipids (Scanu, 1996).

The bovine and human LPLs have similar properties, chemical composition and subunit size (Olivecrona and Bengtsson, 1983) and both are glycosylated. The bovine homolog, however, shows some differences from the human one. For instance, it accounts for 478 amino acids residues with a calculated MW of 42kDa, with the

catalytic triad being Ser-162, Asp-186, His-271. The hLPL, on the other hand is 448 amino acids- long [calculated MW of 50kDa (Wion *et al.*, 1987; Antonian, 1985)] and the catalytic triad is made up of Ser-132, Asp-156, His-241 (He *et al.*, 2018). The signal peptide in the bovine homolog is in positions 1-28, while the human one was in positions 1-27.

Furthermore, the bovine LPL has two additional N-terminal amino acids, Asp-Arg and the major heterogeneity is located in the N-terminal five-residue segment. Since the two enzymes investigated showed the same catalytic efficiency, these residues differences should not have any impact on the protein function, although they could result in a slightly different conformation (Bengtsson-Olivecrona *et al*, 1986) (see Figure 5 for the putative structure of the bLPL monomer).

Restriction enzymes trials showed also that the heparin-binding domain is located in the C-terminal domain (Bengtsson-Olivecrona *et al*, 1986). This domain is essential for the binding and the intra- and extra-cellular transport of the enzyme across the endothelial cell barrier (Pillarisetti *et al*, 1996; Wang and Rodrigues, 2015) for both the human and bovine homologs.

However, differences between the human and bovine LPL structures seem not to affect their catalytic activity, which is the same, and also the bovine blood and milk LPLs are reported to show identical characteristics (Downey, 1975).

LPL works mainly on the luminal surface of the vascular endothelium where it hydrolyses TG-rich lipoproteins like chylomicrons and very low density proteins (VLDL) into glycerol and FFAs but it also releases chylomicrons remnants and intermediate-density lipoproteins (IDL) (He *et al.*, 2018; Wang and Eckel, 2009). It acts on the water-lipid surface of emulsions, like all lipases, and it has a stereospecificity for *sn*-1 and *sn*-3, that is it mainly works on the first and third carbon on the glycerol scaffold of TGs (Jensen and Pitas, 1976; Jaeger *et al.*, 1994; Jaeger *et al.*, 1999; Okuda and Fujii, 1968; Somerharju *et al.*, 1978).

Recently, the structure of the human LPL complexed with its anchor has been solved (Figure 6), suggesting that this enzyme could actually work at its best in the monomeric form (Birrane *et al.*, 2018).

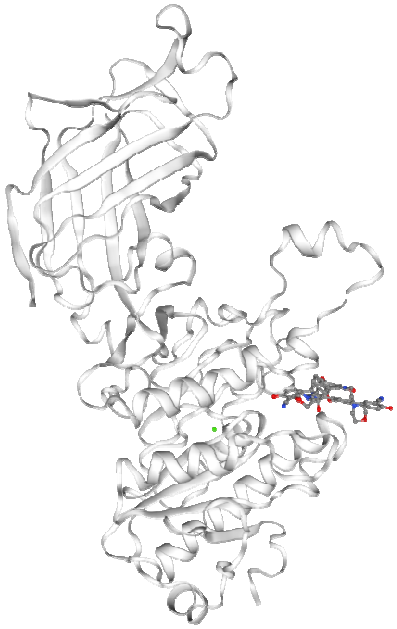


Figure 5 Monomer of the bovine LPL, SWISS model (UniProt).

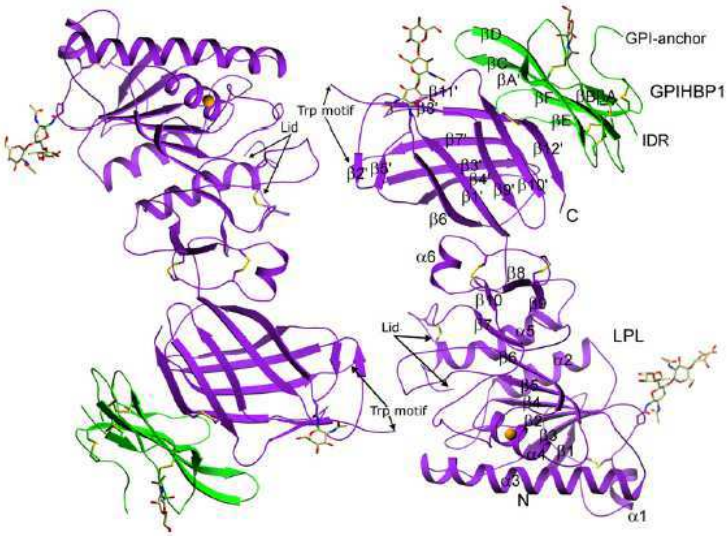


Figure 6 Complex human LPL-GPIHBP1, dimeric (Birrane *et al.*, 2018).

1.4 *Aspergillus nidulans*

Aspergillus nidulans is a filamentous fungus belonging to the *Aspergillus* genus, phylum Ascomycota widely utilized as model organism for both for basic research and for the investigation of strategies aimed at the production of heterologous proteins.

First, a brief overview of this genus will be provided.

1.4.1 Genus: *Aspergillus*

Aspergillus comprises more than 340 species of filamentous fungi, including somepathogenic species, such as *A. fumigatus* and *A. parasiticus*. They are characterised by the generation of filamentous structures named hyphae, which grow polarly and unidirectionally, but also branching tridimentionally into mycelia (Park *et al.*, 2017; Etxebeste and Espeso, 2019). Hyphae could generate conidiophores which bear and release asexual spores called conidia (Adams *et al.*, 1998). Mycelia morphology (colour, powdery state, height etc) varies broadly among strains and are quite useful for their phenotypical classification. Besides, the phenotype of the mycelium correlates meaningfully with their genotype, allowing direct observations on the fitness of the strain during experimental studies as reported in Adams *et al.* (1998), Manoli and Espeso (2019) and Noble and Andrianopoulos (2013).

Most *Aspergillus* species were thought to have only the asexual state and *A. nidulans* was the first in which a sexual stage was observed, leading the way to many others, like *A. fumigatus* (Geiser, 2009).

As for their metabolism and cultural needs, Aspergilli are aerobes and chemoheterooganotrophic as they use O_2 as the final electron acceptor in the conversion of exogenous sources of carbon to energy. What's more appealing is that they can degrade and metabolise various biopolymers, such as starch, hemi-cellulose, pectin, xylan and proteins thus allowing cultivation on cheap and renewable media (Meyer *et al.*, 2010).

The biotechnological potential of the *Aspergillus* genus acquired growing interest among researchers allowing to the continuous development of techniques and strategies to be applied in several research fields and industry (Bennet, 2010; Meyer *et al.*, 2010).

Aspergilli are biotechnologically interesting both for their metabolic versatility and for the wide range of environmental conditions at which they grow (T = 10- 50 °C, pH = 2-11, salinity = 0-4%, activity water = 0.6-1, under oligotrophic and nutrient rich conditions (Meyer *et al.*, 2010)).

Some *Aspergillus* species can produce mycotoxins of major concern like aflatoxins (*A. parasiticus*, *A. flavus*), patulin, citrinin, gliotoxin (*A. terreus*) or ochratoxin (Bennet and Klich, 2003) while *A. nidulans* produces sterigmatocystin, the penultimate precursor of aflatoxin (Yu and Leonard, 1995). Other *Aspergillus* species, however, produce a wide variety of other, often beneficial, products as summarized in Figure 7.

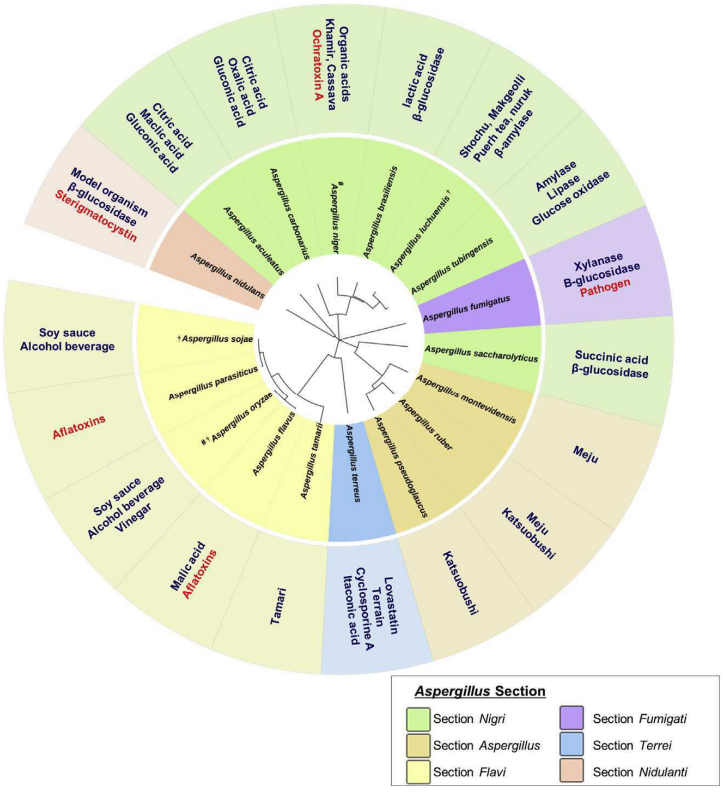


Figure 7 Taxonomy, phylogeny, and key applications/products of industrially important *Aspergillus* species (Park *et al.*, 2017).

Strains used as cell factories usually have the GRAS status (Generally Regarded As Safe) because they are non-pathogenic. Nonetheless, it is important to screen thoroughly the potential of each strain to produce mycotoxins (Frisvad *et al.*, 2007; Houbraken *et al.*, 2014).

A.niger and *A. oryzae* are the most exploited producers thanks to the high yields they allow (MacCabe *et al.*, 2002). *A. oryzae* is used mainly in food fermentations, especially in Asian fermented beverages (Park *et al.*, 2017), but has been also used as host for commercial production of industrial enzymes like lipases, lactases and proteases (Archer, 2000; Pariza and Johnson, 2001).

1.4.2 Species: *Aspergillus nidulans*

A.nidulans, also known as *Emericella nidulans* in the sexual form, has a relatively small genome of 30Mb, divided into 8 linkage groups (chromosomes) and with only few repetitive elements and, as already mentioned, it exhibits both asexual spores and sexual ascospores (Todd *et al.*, 2007).

Pontecorvo's early works (1953) triggered an interest in *A. nidulans*' genome that led to huge amounts of genomic data available and the consequent development of well-established related genetic and molecular tools (Park *et al.*, 2017). Besides, the interaction between genomic information and the newly developed molecular techniques allowed to achieve numerous selection markers and several different auxotrophic strains (strains that cannot autonomously synthetise specific essential molecules like riboflavin) (Dohn Jr *et al.*, 2018). Being one of the most studied species *Aspergillus nidulans* is recognized as model organism for fundamental research investigations like spore development, cell cycle, cell polarity and apical growth, gene function, DNA repair, metabolism, signal transduction, secondary metabolism, sexual and asexual development and pH control (Park *et al.*, 2017; Todd *et al.*, 2007)**.** Moreover, it is already being often used as host for the production of heterologous proteins, like the endoglucanase beta-glucosidase (Kumar *et al*, 2016; MacCabe *et al.*, 2002).

A. nidulans exhibits apical growth of germinated conidia or binucleate ascospores with the formation of multicellular hyphae, which make up the characteristic mycelium. Vegetative hyphae differentiate by asexual development to produce spores (conidia, Figure 8A) on specific structures called conidiophores (Figure 8B). At the base of the club-shaped conidiophore it can be found the foot cell (Figure 8C) which can be L or T shaped and is connected by porous septa to the other hyphae. At the head of the conidiophores primary sterigmata (metulae) are generated and give rise to secondary sterigmata (phialides, Figure 8 D), which in turn, spring the actual conidia [Lier Bettina, 2017,Bachelor Thesis, University of Natural Resources and Life Sciences (BOKU), Vienna].

Figure 8 *Aspergillus nidulans* SAA111 strain, optical microscopy, magnified 100X. The arrows indicate: **A** spores, **B** conidiophore, **C** foot cell, **D** phialides.

Sexual development occurs by the production of nurse cells called Hülle cells (nutrient-providing cells for cleistothecia) or sclerotia (wintering structures) (Houbraken *et al.*, 2014; Samson *et al.*, 2014) and closed sexual fruiting bodies (cleistothecia) containing sexual diploid spores (ascospores) grouped within asci. Each cleistothecium contains more than 10,000 ascospores that are the meiotic progeny of a single hypha (Todd *et al.*, 2007). As can be observed in Figure 9, in the *A. nidulans* life cycle there can be also a third way of replication: the parasexual stage, beginning with two vegetative hyphae from two different individuals merging into a heterokaryon to form a diploid cell which can later take the sexual or asexual route.

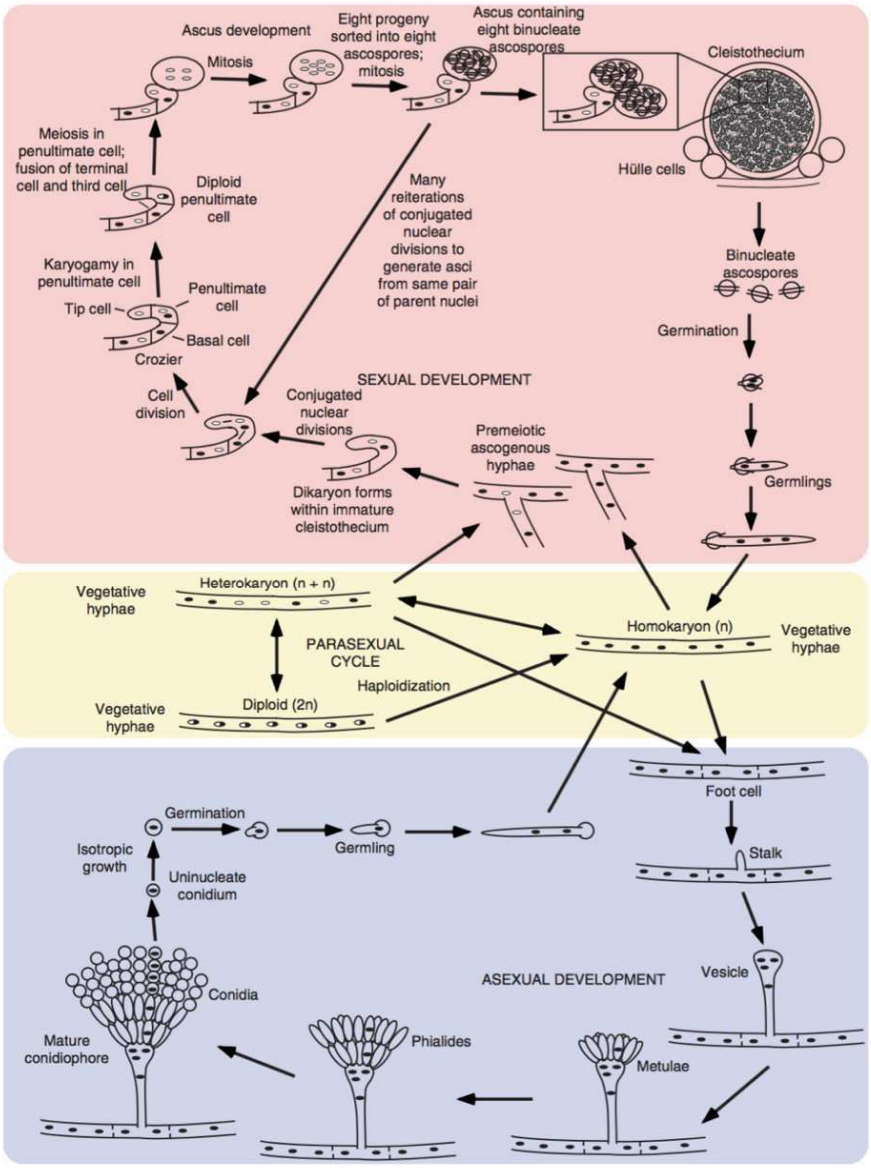


Figure 9 Life cycle of *Aspergillus nidulans* (Todd *et al.*, 2007).

2. WORK AIM

The bovine *lipoprotein lipase* is one on the main milk lipases and so far its role in the milk “tissue” has not been properly investigated. The aim of this work is to establish a new system to produce bLPL in a cheap and efficient manner in order to investigate its structural and functional characteristics. This project will add knowledge to the bLPL role in milk and, in particular, to its implication in the phenomenon of milk rancidity for downstream preservation treatments. Another reason to investigate the structure of bLPL is its similarity with the human homolog involved in a rare disease, the Familial Chylomicronemia Syndrome (FCS).

3. MATERIALS

3.1 Strains and plasmids

In the following tables, the strains and the plasmids utilized in this work are indicated.

Table 1 Strains utilized in this work.

Strain	Genotype	Company
Escherichia coli DH5α	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd* R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
Aspergillus nidulans SAA111	*veA1; biA1;* Δ*argB::trpC; riboB2; pyroA4; wA3*	Kindly donated by Prof. Joseph Strauss (BOKU, Vienna)

Table 2 Vectors utilized for bLPL expression (AlcA plasmid) and as bLPL sequence source (bLPL plasmid).

AlcA plasmid kindly donated by Joseph Strauss (BOKU, Vienna)
bLPL plasmid (LPL Bovine_pcDNA3.1(+), GenScript; handled and stored according to manufacturer's instructions)
gLPL plasmid (LPL Gallus_pcDNA3.1(+), Genscript; handled and stored according to manufacturer's instructions)

AlcA plasmid harbours several restriction sites, as shown in Figure 10. *BamHI* and *EcoRI* were chosen as cloning sites. Moreover, two origins of replication, one for *E. coli* and one for *Aspergillus* spp. are present in the plasmid. Ampicillin resistance selection marker and the *argB* gene (GenBank M29819.1), which complements the arginine auxotrophy in the fungus, were used for plasmid propagation in *E. coli* and the selection of *Aspergillus* transformants, respectively. The promoter of the *Alcohol*

dehydrogenase (*alcA*) gene (GenBank M16196.1) was chosen to drive the heterologous gene expression and the *trpC* polypeptide gene (GenBank U24705.1) as the *Aspergillus* gene terminator. The plasmid was propagated in *E.coli*DH5αstrain and extracted by means of the Eurogold Plasmid Miniprep Kit (EuroClone), according to the manufacturer's protocol.

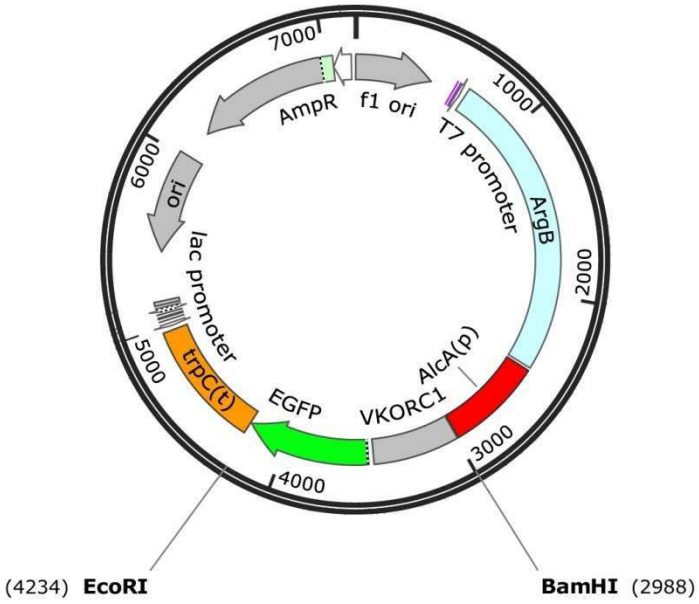


Figure 10 AlcA plasmid map. AlcA promoter and trpC terminator are respectively indicated in red and orange. The *Aspergillus* selection marker *argB* is indicated in light blue. The fragment VKOC1-eGFP was replaced by bLPL nucleotide sequence.

The bovine *lipoprotein lipase* (bLPL) DNA sequence was purchased from GenScript. The plasmid harbouring bLPL was used as vector in *E.coli* for propagation and a 1:30 dilution of the same was utilized as template for PCR purposes.

3.2 Media, solutions and buffers

Growth media for *Escherichia coli*:

All reagents were purchased from SIGMA, except when specified.

LB (Luria Bertani) broth per L:

Table 3 Luria-Bertani Medium

Reagent	Amount
Tryptone	10g
Yeast Extract	5g
NaCl	10g

For solid LB medium, 20g/L of agar was added to the liquid broth.

- SOC (Super Optimal Broth with Catabolite Repression) medium- Novagen
- Ampicillin stock 1000X(100mg/ml)

Solutions for preparation of *E. coli*DH5α competent cells:

- CaCl2·2H2O100mM-Merck
- $CaCl_2 \cdot 2H_2O$ + $MgCl_2$ x6H_2O solution ca. 100 mM

Growth Media for *Aspergillus nidulans*:

Aspergillus Minimal Medium (AMM), agar:

Table 4 AMM composition

Reagent	amount
Agar	2%
Salts	1X
Trace elements	1X

Salts 20X, per L:

Table 5 Salts solution for *Aspergillus* growth

Reagent	**Amount**
KCl	10.4g
KH2PO4	16.3g
K2HPO4	20.9g

Traces 1000X:

Table 6 Trace elements added to the culture medium for *Aspergillus* growth.

Reagent	**Amount(g/L)**
$ZnSO_4 \cdot 7H_2O$	22
H_3BO_3	11
$MnCl_2 \cdot 4H_2O$	5
$FeSO_4 \cdot 7H_2O$	5
$CoCl_2 \cdot 5H_2O$	1.6
$CuSO_4 \cdot 5H_2O$	1.6
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.1
Na_2EDTA	50

Complete Medium (CM), agar, per L:

Table 7 Complete Medium composition.

Reagent	**Amount**
Salts	20X
Traces	1000X
$MgSO_4$	200X
Casein Hydrolysate	2g
(D) glucose	10g
Peptone	2g
Yeast Extract	1g
Agar	1.5%
vitamin mix	1x

Vitamin Mix 100x:

Table 8 Vitamin mix composition for complete medium.

Reagent	**Amount (mg)**
p-aminobenzoic acid	20
thiaminHCl	50
biotin	1
nicotinic acid	100
calcium p-pantothenate/pantothenic acid calcium	200
Pyridoxine - HCl	50
Riboflavin	100

Top agar

Table 9 Top agar used to grow transformants.

Reagent	amount
Sucrose	1M
agar	1%
supplements	See table11

Supplements:

Table 10 Supplements added to the AMM.

Reagent	amount
Pyridoxine chlorhydrate	1X
Biotin	1X
Arginine	1X
NH4+ tartrate	1X
MgSO4	1X
Riboflavin	1X
Glucose	10%

Tween 20, 0.1%

Transformation of *Aspergillus nidulans*

- Glucanex/ lysis enzyme from *Trichoderma harzianum* – Sigma
- KCl 0,7M
- Spectra/Mesh woven Filters (mesh opening 10µm) – Millipore

Table 11 Tn1 and Tn2 solutions.

Tn1		**Tn2**	
Reagent	**concentration**	**Reagent**	**concentration**
KCl	0.7M	PEG	60%
$CaCl_2$	50mM	Tris-HCl	100mM, pH 8.0
		$CaCl_2$	50mM

3.3 Molecular analysis

Agarose gel:

Table 12 Agarose gel.

Reagent	**amount**
Agarose –invitrogen	1%
TAE	1X
GelRed 10000X/Sybr Safe 5000X	1X

TAE (for 500ml):

Table 13 TAE 50x. The working solution was eluted to 1X.

Reagent	**amount**
Trizma base	121g
Acetic acid 100%	28.55ml
EDTA	0.5M pH= 8.0 50ml
DW	to volume

- Gene Ruler 1kb- Thermoscientific
- Loading Dye 6X - Thermoscientific

Ligation:

- T4 ligase – Invitrogen
- T4 ligase buffer – Invitrogen

Plasmid extraction from *E. coli*:

- Eurogold plasmid miniprep kit –Euroclone

DNA purification:

- GeneJet Purification Kit – Thermoscientific
- Isopropanol

Solutions for genomic DNA extraction from *A. nidulans*:

Table 14 *Aspergillus* genomic DNA extraction solutions.

Buffer 1	**amount**	**Buffer2**	**amount**	**Buffer 3**	**amount**
Resuspension		Lysis		Neutralization	
Tris-HCl pH 8	50mM	NaOH	200mM	Potassium Acetate pH 5.5	3.0M
EDTA pH 7.5-8	10mM	SDS	1%		
RNAse A	100µg				
DW to volume		DW to volume		DW to volume	

PCR:

- Green Kit: Green GoTaq Flexi – PROMEGA
- Red Kit: VWR Red Taq DNA Polymerase Master Mix – VWR LIFE SCIENCE
- Phusion Kit: Phusion High-fidelity DNA Polymerase – Thermoscientific

Primers:

Table 15 Primers used for the PCR reactions.

	Tm (°C)	Working concentration	Sequence
bLPLBamHI_fw	79.1	10µM	5'-GACGGATCCATGGAGAGCAAGGCCCTACTTC
bLPLCstrepEcoRI_rv	90	10µM	5'-GCCGAATTCTTTTTCGAACTGCGGGTGGCTCCAGGCAGAGCCAGACTTTCTATTCAG
bLPLint_rv	65.2	10µM	5'-CACATCTCCSSGGCCTCTCTC
bLPLstart_rv	72	10µM	5'-CTCTGCAGGCACACGCTCAGAG
hLPLint_fw	72.6	10µM	5'-GGTGGACTGGCTGTCACGGG
hLPLint_rv	60.5	10µM	5'-CACATCTCCAAGTCCTCTCTC
AlcAp_up_fw	51.7	10µM	5'-TCTGCGATCGTCCATAAC
trpC_rv	62.4	10µM	5'-CGATAAGCTTGATATCGAATTCC

Restriction enzymes:

Table 16 Restriction enzymes.

Enzyme	Buffer	Incubation	Restriction site sequence
EcoRI	EcoRI buffer	2h x 37°C	5'-GAATTC-3'
BamHI	BamHI buffer	2h x 37°C	5'-GGATCC-3'
HindIII	Red 1x/TANGO 1X-2X	2h x 37°C	5'-AAGCTT-3'
XhoI	Red1x/TANGO 1X-2X	2h x 37°C	5'-CTCGAG-3'

Table 17 Centrifuges.

Centrifuge	**Company**
BiofugeprimoR	Heraeus
Centrifuge 5424	eppendorf
Genofuge 24D	TECHNE

- Optical Microscope: Reichert-Biovar
- Immersion Oil – FLUKA

Table 18 Spectrophotometers.

Spectrophotometer	**company**
UV-1700 Pharma Spec	SHIMADZU
DU800	BeckmanCounter

Spettrofluorimeter:Synergy HT- Biotek

SDW water prepared with sterilised MilliQ water at 121°C for 20mins.

3.4 Proteomics

3.4.1 Crude extracts

In the present paragraph all the necessary reagents and equipment utilized to prepare crude extracts are listed:

- Liquid nitrogen
- Miracloth membrane- MerkMillipore
- Glass beads 230-300 μm
- Plant PIC (proteinase inhibitor complex)
- Fungi PIC(proteinase inhibitor complex)
- Low salt extraction buffer 0.2M, pH 7.2(see Table 19)
- High salt extraction buffer 1M, pH 7.2 (see Table 20)

Table 19 Low salt extraction buffer used.

Reagent	**concentration**
Tris HCl	20mM
NaCl	200mM
Glycerol	30%
MilliQ water	To volume

Table 20 High salt extraction buffer used for crude extracts to be passed only in the Strep-Tactin® column.

Reagent	**amount**
Tris-HCl	20mM
NaCl	1M
Glycerol	30%
MilliQ water	to volume

3.4.2 Bradford Assay

- Bovine Serum Albumin (BSA)
- Bradford reagent – Sigma Aldrich 0.1-1.4 mg/ml

3.4.3 SDS-PAGE

4X sample buffer:

Table 21 4X Sample buffer preparation.

Reagent	**amount**
Glycerol	40%
Tris-HCl	250mM
β-mercaptoethanol	2.9M
Bromophenol Blue	0.03%
SDS	8%

Laemmli 12%:

Table 22 LAEMMLI polyacrylamide gel, 12%.

Running gel (2 gels)		**Stacking gel (2 gels)**	
Reagent	**amount**	**Reagent**	**amount**
Running buffer	2ml	Stacking buffer	1ml
MilliQ water	4ml	MilliQ water	3.5ml
Acrylamide(30% stock)	4ml	Acrylamide (30% stock)	0.5ml
APS 10%	50µl	APS 10%	30µl
Temed	5µl	Temed	3µl

Acrylamide 30% stock: 29.24% acrylamide, 0.76% bis-acrylamide, 70% water

Running buffer:

Table 23 Running buffer utilized for SDS PAGE run of 12% polyacrylamide gel.

Reagent	**amount**
Glycine	14.4g
Trizma base	3.02g
SDS	1g

Tris-glycine gel:

Table 24 Tris glycine acrylamide gel for high salt extracted samples.

Running gel (2 gels)		**Stacking gel (2 gel)**	
Reagent	**amount**	**Reagent**	**amount**
Glycerol	2g	MilliQ water	4.15ml
MilliQ water	5.33ml	Acrylamide	0.5ml
Gel buffer	5ml	Gel buffer	1.55ml
Acrylamide 49.5%	3.05ml	APS 10%	50µl
APS 10%	60µl	Temed	3µl
Temed	6µl		

- Anode buffer (Table 25)
- Catode buffer (Table 26)

Table 25 Tris-glycine anode buffer.

Reagent	**amount**
Tris- Hcl pH 8.9	0.2M

Table 26 Tris-glycine catode buffer.

Reagent	**amount/500ml**
Tris-HCl pH 8.25	6.06g
Tricine	8.96g
SDS	0.5g

3.4.4 Coomassie staining

Staining solution.

Table 27 Staining solution for the acrylammide gels.

Reagent	**concentration**
Coomassie Brilliant Blue (CBB) R250	0.1%
Methanol	50%
Acetic acid	10%
MilliQ water	to volume

Destaining solution 1.

Table 28 First destaining solution.

Reagent	**concentration**
Distilled water	to volume
Acetic acid	10%
Methanol	50%

Destaining solution 2.

Table 29 Second destaining solution.

Reagent	**concentration**
Distilled water	to volume
Acetic acid	7%
Methanol	5%

3.4.5 Western blotting

Trans-Blot® Mini Nitrocellulose Transfer Packs- BioRad

Primary antibody: anti-strep (Precision protein streptactin HRP conjugated – Biorad) 1:1000 in blocking solution

Primary antibody: anti-LPL (LPL 5D2 sc-73646 Mouse monoclonal IgG- Santa Cruz Biotechnology) 1:10000 in blocking solution (see Table 32)

Secondary antibody: anti-mouse (anti mouse IgG HRP conjugated - R&D Systems) 1:10000 in blocking solution

TBS

Table 30 Tris Buffer solution for washings.

Reagent	**concentration**
Tris-HCl, pH 7.6	20mM
NaCl	137mM

TBS-T

Table 31 TBS-T solution used for membrane washings.

Reagent	**concentration**
TBS	1X
Tween 20	0.1%

Blocking solution

Table 32 Blocking solution used in the WB and to dilute antibodies.

Reagent	Concentration
TBS-T	1X
BSA	5%

Stripping

Mild stripping solution, pH 2.2

Table 33 Stripping solution, pH 2.2, used to wash WB membranes from antibodies.

Reagent	Amount/L
Glycine	15g
SDS	1g
Tween 20	10ml
MilliQ water	to volume

PBS 100mM, pH 7.2

Table 34 PBS buffer solution.

Reagent	amount/50ml
NaCl	4g
KCl	0.1g
$Na_2HPO_4 \cdot 7H_2O$	1.085g
KH_2PO_4	0.1295g
MilliQ water	to volume

3.4.6 Purification

3.4.6.1 Strep-Tactin® column for Strep-tag affinity chromatography

Strep-Tactin® resin- ibalifesciences

Solutions for the Strep-Tactin® column purification protocol

Table 35 Solutions for the purification protocol with the Strep-Tactin® column.

Washing buffer pH 8		**Elution buffer**	
Reagent	concentration	Reagent	concentration
Tris-HCl	100mM	Tris-HCl pH 8	100mM
NaCl	150mM	NaCl	150 mM
EDTA	1mM	EDTA	1mM
		Biotin	50mM
MilliQ water	to volume	MilliQ water	to volume

3.4.6.2 Heparin-Sepharose column affinity chromatography

Heparin-Sepharose resin.

Tris-based solutions for the Heparin-Sepharose column.

Table 36 Tris-based solutions used in the Heparine-Sepharose purification protocol.

Equilibration/washing buffer pH 7.2		**Elution buffer pH 7.5**	
Reagent	concentration	Reagent	concentration
Tris-HCl	100mM	Tris-HCl	100mM
NaCl	100mM	NaCl	1.5M
EDTA	1mM	EDTA	1mM
MilliQ water	to volume	MilliQ water	to volume

PBS-based solutions for the Heparin-Sepharose column.

Table 37 PBS-based solutions for the Heparin-Sepharose column purification protocol .

Washing buffer pH 7.2		**Elution buffer pH 7.5**	
Reagent	concentration	Reagent	concentration
PBS	20mM	PBS	10mM
		NaCl	1.5M

4 METHODS

4.1 *E. coli*

Escherichia coli DH5αstrain provided by Invitrogen (Genotype: F^{-} φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) was used as host for plasmid propagation. It is an engineered strain improved for transformation efficiency. DH5α cells are sensitive to ampicillin and chemically competent for the internalization of exogenous vectors.

4.1.1 Growth conditions

E. coli strains were grown on LB agar medium at 37°C, while clones selected for the target plasmid were grown on LB in the presence of the Ampicillin, at the same temperature. An overnight incubation was sufficient to obtain colonies. Broth cultures were grown in LB with or without antibiotic with gentle stirring to allow oxygenation.

Strains were stored on plates at 4°C for up to one week. 80% glycerol solution was added to aliquots of broth cultures for long-term storage at -80°C.

4.1.2 Competent cells preparation

The preparation of competent *E. coli* DH5α cells was performed through$CaCl_2$chemical method. Cells from an overnight pre-culture on liquid LB medium were harvested and inoculated in fresh LB medium and grown up to an OD of 0.4-0.6 (3h ca). Cells were then stored in ice and treated with calcium- and magnesium-chloride solutions. Aliquots of competent cells were stored at -80°C and

tested by transforming them with AlcA plasmid or an additional control plasmid (e.g. pUC19).

4.1.3Transformation

Competent cells were transformed by adding the desired plasmid followed by thermal shock, according to protocol in terms of temperature and time. Cells were then incubated at 37°C, slightly shaking for 1 hour and plated on agar LB-Ampicillin medium for overnight growth.

4.1.4 Clone selection

Colonies grown after transformation were replicated on LB agar-Ampicillin. Colony PCR was performed using the primers hLPL_int_fw and bLPL_int_rv (see Table 15) for the amplification of a 451 bp internal fragment of bLPL. Selected colonies showing the amplified sequence were replicated on fresh medium with Ampicillin.

After growth, two clones were selected for further investigations. The plasmidic DNA of each clone was extracted and quantified. A series of amplifications was performed, as follows:

- Amplification of the whole sequence (1461 bp) with the high-fidelity Phusion® Master Mix;
- Amplification of an internal sequence (451 bp) using the GoTaq mix;
- Amplification of a sequence containing the whole AlcA promoter (592bp)using the GoTaq mix.

Mixes and cycles are described in paragraph **4.3.1**.

4.2 *Aspergillus nidulans*

We used the strain SAA111[Genotype: *veA1; biA1; ΔargB:: trpC; riboB2; pyroA4; wA3* (Guerriero *et al*, 2017)], which grows radially forming a white mycelium on AMM andon CM. SAA111 is auxotrophic for arginine due to the deletion of *argB* gene encoding for the ornithine carboamyl transferase. The integration of AlcA plasmid restores the lacking of *argB* wild type gene copy, allowing *Aspergillus* to grow without any exogenous addition of arginine.

Aspergillus nidulans strains were grown on solid and liquid AMM supplemented with pyridoxine chlorohydrate, biotin, riboflavin and arginine, in order to complement the auxotrophic requirements, and with the addition of magnesium. Ammonium tartrate and glucose were added as nitrogen and carbon sources, respectively (except were explicitly stated otherwise).

Transformed protoplasts were grown on Top agar containing sucrose as carbon source and all the necessary supplements. Transformants were grown at 37°C for 2-5 days and replicated on CM for genomic stabilization of the integrated fragment Figure 11).

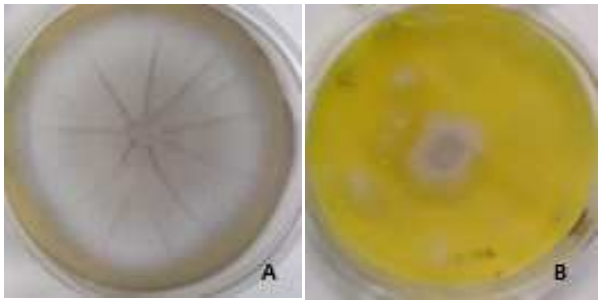


Figure11 *Aspergillus nidulans* on Complete Medium (panel A) and Minimal Medium (panel B).

4.2.1 Strain revitalization from culture collection

Strains aliquots stored at -80°C were thawed on ice and then plated on fresh AMM. Plates were incubated at 37°C for growth. Strains were then replicated on new plates by picking conidia or streaking spore solutions.

4.2.2 Spore solution and cryopreservation

Spores were harvested by adding Tween 20 0.1% on the culture directly on agar plate. Then the Tween-spore suspension was collected and utilized for the inoculum or for storage.

Spore solutions were stored up to one month at 4°C. For long-term storage at -80°C, spore solutions were mixed to glycerol 80% solution in the 1:1ratio.

4.2.3 Liquid culture and harvesting of biomass

Liquid cultures were grown in broth AMM with the proper supplements, at 37°C and 160-180 rpm. Later biomass was harvested by filtration through Miracloth, and rapidly frozen in liquid nitrogen. Collected biomass was then stored at -80°C in aluminium foils.

4.2.4 Light microscopy

Cell fitness, osmolar stress and morphology as well as protoplasts production and transformants have been observed at the optical microscope Reichert Biovar at 10X, 40X and 100X of magnitude.

4.2.5 Aspergillus transformation and selection

Protoplasts were generated by enzymatic digestion of the fungal cell wall with Glucanex, the *Trichoderma harzianum* lysing enzyme. The working solution consists of 0.8g of the lysing enzyme in 20ml of KCl 0.7M, harvested by filtration with the spectra woven filters and promptly transformed according to protocol using the solutions Tn1 and Tn2 (see Tables 11), then plated on Top agar (see Table 9) without arginine and incubated for 3 to 5 days at 37°C.

Three to five days after transformation, the first transformants were distinguishable from the background. Transformants were picked and replicated on CM to stabilize the sequence integration. Grown transformants were then replicated on AMM and,

when *Aspergillus* has sporulated, spores are harvested as spore solutions and used to extract the genomic DNA from the relative transformants and to make stocks to store at -80°C.

Genomic extracts were quantified, analysed on agarose gel and utilized as templates for detection of integrated bLPL *via* PCR.

4.2.6 Aspergillus transformants induction

The AlcA promoter is a strong inducible promoter (Felenbok, 1991) regulated at the transcriptional level. It can be induced by ethanol and other carbon sources like threonine and ethyl methyl ketone (Creaser *et al.*, 1984) while it is repressed by glucose, which is the microorganism preferred carbon source.

Selected transformants were, therefore, grown in liquid AMM both for 25 hours with 1% ethanol as carbon source or for 16-18 hours with glucose, washed and then induced for 25h with 1% ethanol. At the end of the incubation time, they were harvested for subsequent steps.

4.3 Molecular methods

4.3.1 Molecular biology analyses

DNA samples from clones where extracted with the Eurogold plasmid miniprep kit (see Materials, Paragraph 3.2).

1- PCR with high-fidelity polymerase Phusion® Master Mix and utilized primers set:

- bLPLBamHI_fw
- bLPL C-strep EcoRI_rv

amplified fragment: 1461bp (whole)

Mix:

DNA template 1µl

Phusion® mix 10µl

primer1 1µl

primer2 1µl

SDW 7µl

tot 20µl

Cycle

98°C 30’’

98°C 30’’

65°C 30’’ } x25 cycles

72°C 30’’

72°C 7’

4°C ∞

2- GoTaq Green, primer set:

- hLPLint_fw
- bLPLint_rv

amplified internal fragment: 451bp

3-GoTaq Green, primer set:

- AlcAup_fw
- bLPLstart_rv

amplified fragment: 592bp comprising the promoter.

Sometimes the promoter region was amplified with the primer set:

- AlcAup_fw
- bLPLintrv

with T_m= 57°C, achieving an amplified sequence of 1099bp.

2- and 3- Mix:

DNA template 1µl

5xGreenBuffer 5µl

dNTPs 1µl

primer1 2µl

primer2 2µl

MgCl2 2µl

SDW 11.5µl

GoTaq0.5µl

Tot 25µl

Cycle

95°C	5’	
95°C	30’’	x25 cycles
60°C	30’’	
72°C	40’’	
72°C	7’	
4°C	∞	

Amplification with the Red Taq was carried out for all the above cited sequences, with the same cycles as the green GoTaq (according to manufacturer’s instructions), with the following mix:

DNA template 3µl

Primer1 1µl

Primer2 1µl

Red Mix 20µl

Tot 25µl

Amplified fragments are then prepared with the loading dye if coming from the PCR with the Phusion® master mix and visualised and checked on agarose gel with 1% run at 50-80 Volt along with a reference ladder.

4.3.2 Restriction and ligation

Restriction of both AlcA plasmid and bLPL-Cstrep was performed with the enzymes *EcoRI* and *BamHI* according to the specifics in Table 17 (Materials, Paragraph 3.3). Due to early problems in its excision (Results 6.3),bLPL plasmid was further digested with the enzymes *EcoRI*, *BamHI*, *HindIII*, *XhoI* and *HindIII* together with *XhoI* with the TANGO 1X buffer (according to Table16) in order to achieve a restriction profile indicative of the correct construction of the plasmid scaffold that was bought.

Once both the AlcA plasmid and the bLPL-Cstrep sequence were correctly digested they were ligated with the T4 ligase according to manufacturers' protocol.

4.3.3 Colony PCR

A pellet of cells is added 20µl of SDS and incubated at 95°C for 15 minutes to loosen the membrane lipidic layers. 1µl of this suspension id used to perform PCR. Colony PCRs usually come out with aspecific bands.

4.3.4 Genomic DNA extraction from *Aspergillus nidulans*

DNA samples were extracted through DNA mini preparations from spore solutions. Spores were harvested by centrifugation and the pellet was re-suspended in sterile water. Buffer 1 and 2 (see table 14 for composition) were added to each aliquot and then samples were incubated at 65°C for 20 minutes. After the incubation, buffer 3 was added and samples were centrifuged, and DNA was precipitated by using isopropanol. The extracted DNA was then washed with ethanol 70% and then re-suspended with SDW.

4.3.4.1 PCR and gel electrophoresis for transformants selection

All amplifications of fragments from the genomic DNA of *Aspergillus* transformants use 2µl of sample DNA (and one less of water), 3µl when using the Red Master Mix.

Phusion® mix amplification of a 1382bp fragment with the primers:

- AlcAp_up_fw
- hLPL_int_rv/bLPL_int_rv

performed with T annealing = 55°C and elongation time = 15’’.

This checking point had to be changed because of the struggles with the primer bLPL_Cstrep_EcoRI_rv when applied to the amplification of the transformant genomic DNAs.

4.4 Proteomics

4.4.1 Crude extracts

The frozen biomass was grained in liquid nitrogen by using mortar and pestle. The extraction buffer was added in the ratio 1ml to 300mg of biomass. Fungal and plants PICs were added to the grained biomass together with 50µl of glass beads. Samples were vortexed for one minute and stored in ice for 5 minutes for three cycles. Samples were then centrifuged for 5 minutes and the supernatant was collected.

4.4.2 Bradford Assay

4.4.2.1 Standard curve

500µl aliquots of BSA (bovine serum albumin) were prepared at the following concentrations:

1000µg/µl

500µg/µl

100µg/µl

10µg/µl

5µg/ml

Then 500µl of Bradford reagent was added and read at λ=595nm. The standard curve was drawn using BSA concentrations as abscissae and absorbances as ordinates. The resulting equation of said curve allows finding samples concentrations.

4.4.2.2 Sample quantification

10µl of crude extracts were added to 490µl of MilliQ water and 500µl of Bradford reagent and, after few minutes their absorbance was measured, and their concentration evaluated as already described. Knowing the proteic concentration of the crude extract, they were titrated to 20mg to be used for the electrophoresis step.

4.4.3 SDS- PAGE

The Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a method used to separate proteins based only on their MW. Proteins tend to migrate with different individual behaviours relating to interactions between their shape and gel latex and/or their charge and the applied electric field. SDS prevents these phenomena. It is, in fact, a strong denaturing agent, making proteins lose their tridimensional conformation. It also confers molecules a net negative charge.

4.4.3.1 Samples preparation

Samples were prepared adding the 4X sample running buffer (see Table 21) and MilliQ water to volume. Mixtures were then boiled at 100°C for 5minutes and stored at -80°C or used right away.

Samples, assumed to have very low protein concentrations and already in liquid form, like purification washings and eluates, were just added with the 4X sample running buffer, boiled and loaded in the gel wells.

4.4.3.2 LAEMMLI protein gel

The gel was prepared according to the standard protocol (Table 22) and it was run at 30-50eV. When both Coomassie staining and Western Blotting were needed two gels were loaded and run in parallel for standardization sake.

4.4.3.3 Tris-glycine protein gel

This gel preparation was used for high salt extracted samples. It was run at 20eV for the stacking gel and 50eV for the running gel, as per standard protocol.

4.4.4 Coomassie staining

Once the SDS-PAGE run was completed, the polyacrylamide gel was detached from the glasses and put in the staining solution for 20 minutes. The gel was then treated with the destaining solution 1 (Table 28) and then with the second destaining solution (Table 29) while slowly shaking until complete gel decoloration.

The Coomassie-stained gel was then scanned with the GS-800 Calibrated Densitometer and acquired through the software Quantity One.

4.4.5 Western blotting

WB is an immunochemical assay where proteins transferred from the SDS-PAGE gel to a nitrocellulose membrane are recognised by specific antibodies. Proteins were transferred with the BIORAD Trans-Blot Turbo – Transfer System and proteins were detected either with the anti-LPL antibody which requires the secondary conjugated anti-mouse antibody or the already peroxidase-conjugated anti-Strep antibody. Resulting membranes were treated with peroxide and luminal at 1:1 ratio and the results were acquired at the CHEMIDOC (Biorad).

Membrane stripping

Membranes were treated with anti-Strep antibody, stripped with the stripping solution (Table 33), washed with PBS (Table 34) and T-TBS (Table 31) and further treated with the anti-LPL antibody. Both antibodies were used to avoid taking into consideration aspecific bands.

4.4.6 Purification

bLPL was purified from the crude extracts by means of affinity chromatography. Crude extracts were passed through a resin with high affinity for the protein of interest. Flow throughs were measured at the fluorimeter at λ=280nm. After several washes, when the $Abs_{|280nm} \rightarrow 0$, bound proteins were eluted either applying a high salt concentration or a high concentration of the same- or a higher- affinity molecule (Lehninger).

4.4.6.1 Strep-Tactin® column affinity chromatography

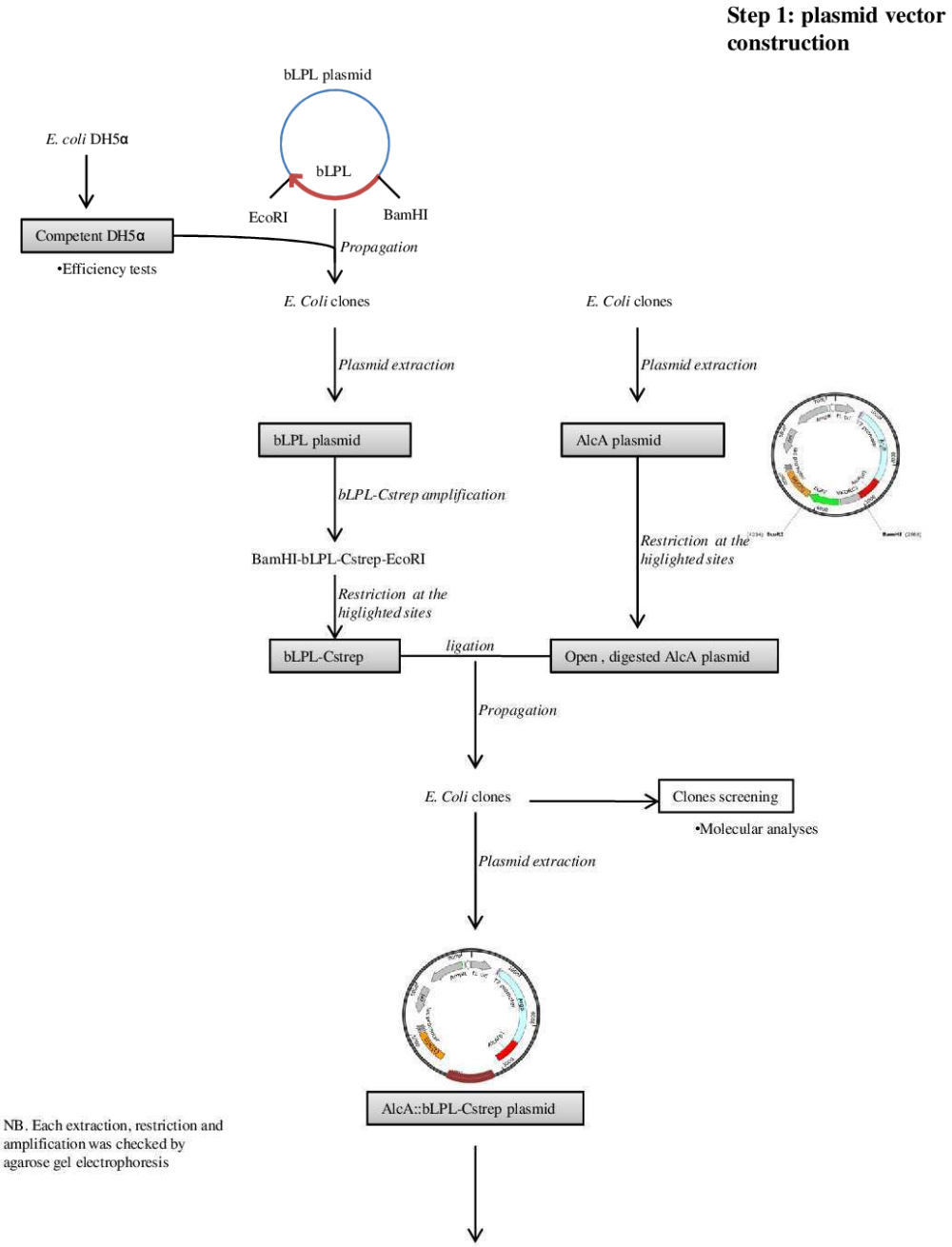
This column exploits the high affinity of the Strep-tag® attached to the protein in C terminal and Strep-Tactin®, an engineered strepavidin. According to the manufacturer's manual, the recombinant protein is poured into the column where it binds with Strep-Tactin® and is later eluted with biotin, which binds competitively to the Strep-Tactin®.

The above described methods were performed with the sequential *cosecutio* logic deligned in the Design of Experiment (Chapter 5, Figure 11).

4.4.6.2 Heparin-Sepharose column affinity chromatography

The sepharose column has got heparin bound to the resin beads. The method exploits its reaction with the LPL heparin-binding domain. The protein was eluted with 1.5M NaCl as already described in several papers (Hernell and Olivecrona, 1974; Tajima *et al.*, 1984; Hajashi *et al.*, 1986).

5. Design of Experiment



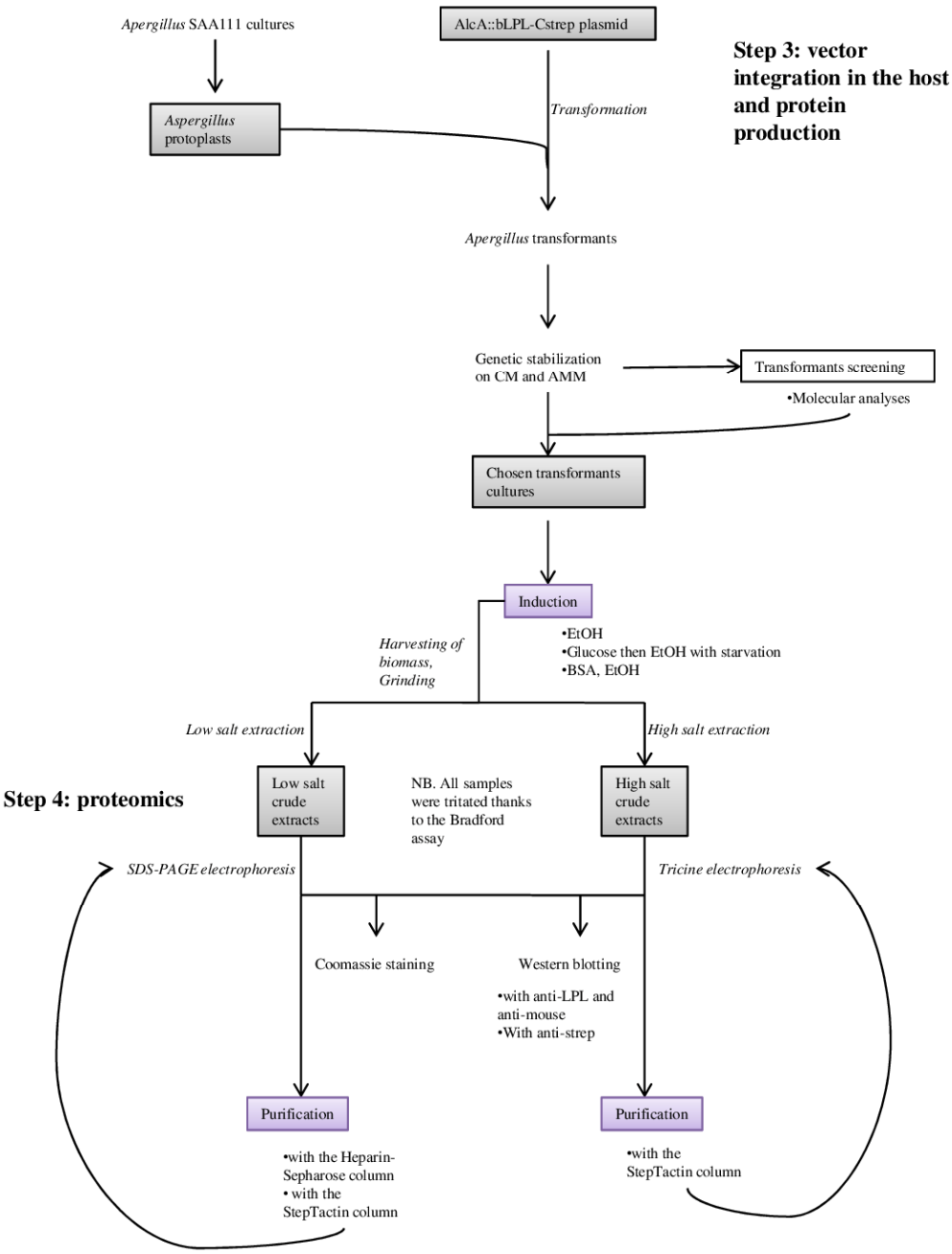


Figure 12 The Design of Experiment sums up the sequential logic of the present work.

6. RESULTS

6.1 *E. coli* results

6.1.1 Competent cells preparation

Competent cells were prepared twice according to Paragraph 4.1.2 and stored at -80°C with the addition of glycerol. Subsequently, their efficiency was tested by transforming them with the same amount (70ng) of AlcA plasmid. Colony counts were carried out after overnight incubation. Results in Table 38 show that the prepared competent DH5α cells were healthy and able to thrive after transformation.

Table 38 DH5α efficiency test results. The pedices $_{i}$ and $_{ii}$ represent cells that were prepared the first and second time, respectively.

	DNA	**Colonies**	**UFC/ml**
DH5α$_{i}$::AlcA	70ng	80	800
DH5α$_{ii}$::AlcA	70ng	>300	>3000

6.1.2 Transformation

The transformation of *E.coli* with the AlcA::bLPL-Cstrep plasmid was successful resulting in an average of 30-40 colonies per Petri dish grown on agar LB with 0.1% ampicillin, as shown in Figure 13 with red arrows.

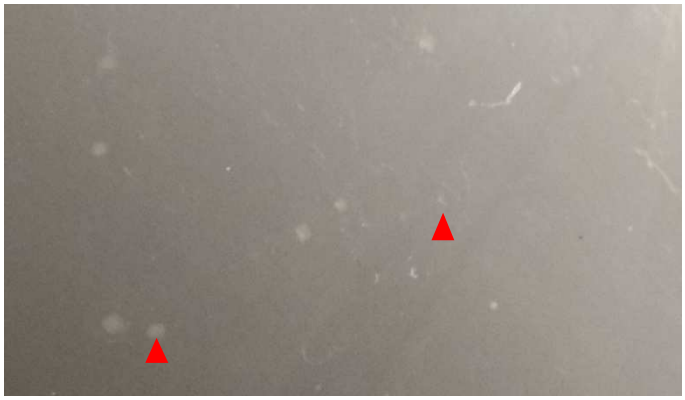


Figure 13 *E. coli* clones after transformation with the AlcA::bLPL-C-strep plasmid.

Developed colonies have been replicated on master plates (Figure14) to get enough biomass to extract the DNA necessary for further analyses.

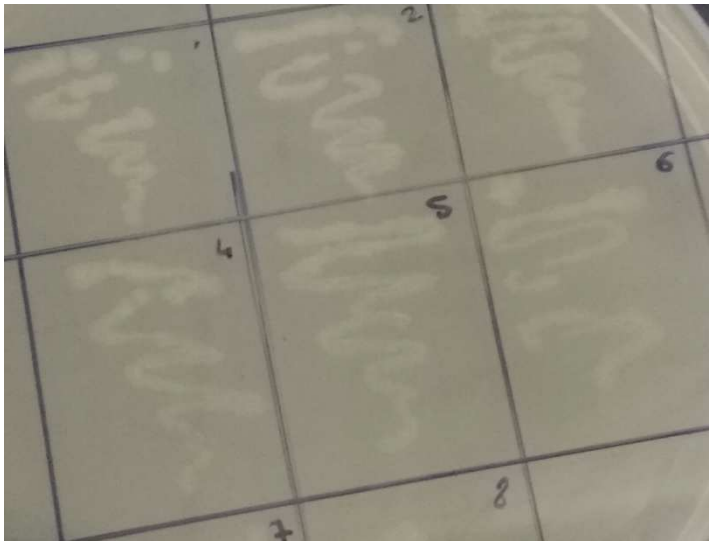


Figure 14 Master palate of *E.coli* clones.

Subsequently some strains have been obeserved at the optical microscope to ascertain cell fitness, as it can be seen in figure 15. Given the dirty background of the lenses some cells have been pointed with red-bordered arrows.

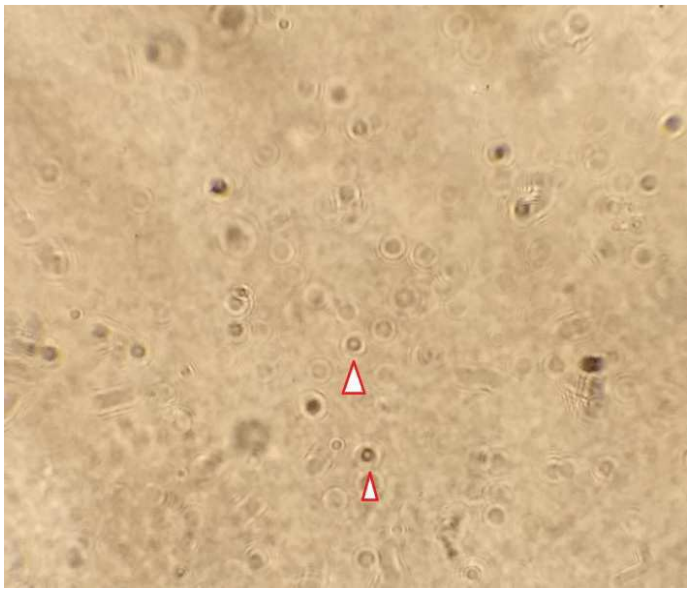


Figure 15 *E.coli* clone 8 at the optical microscope. Magnitude 100X.

As the picture above mentioned shows, the *E. coli* cells in the sample above are quite round but still viable. The slight change in shape may be due to the stress induced by the transformation.

E. coli colonies growth on LB added of ampicillin and thriving of subculture on the same medium suggests entrance of the vector, although it is only a prerequisite but not sufficiently indicative of its presence. Thus, molecular analyses follow.

6.1.3 Clone selection

A rough PCR from the selected colonies amplifying the internal fragment was the first step in the clone selection process. Colony PCR usually gives a lot of aspecific bands, as shown in figure 16. In fact, it is difficult for the primers to anneal when the DNA sequence of interest is in solution with all the cell debris and the genomic DNA.

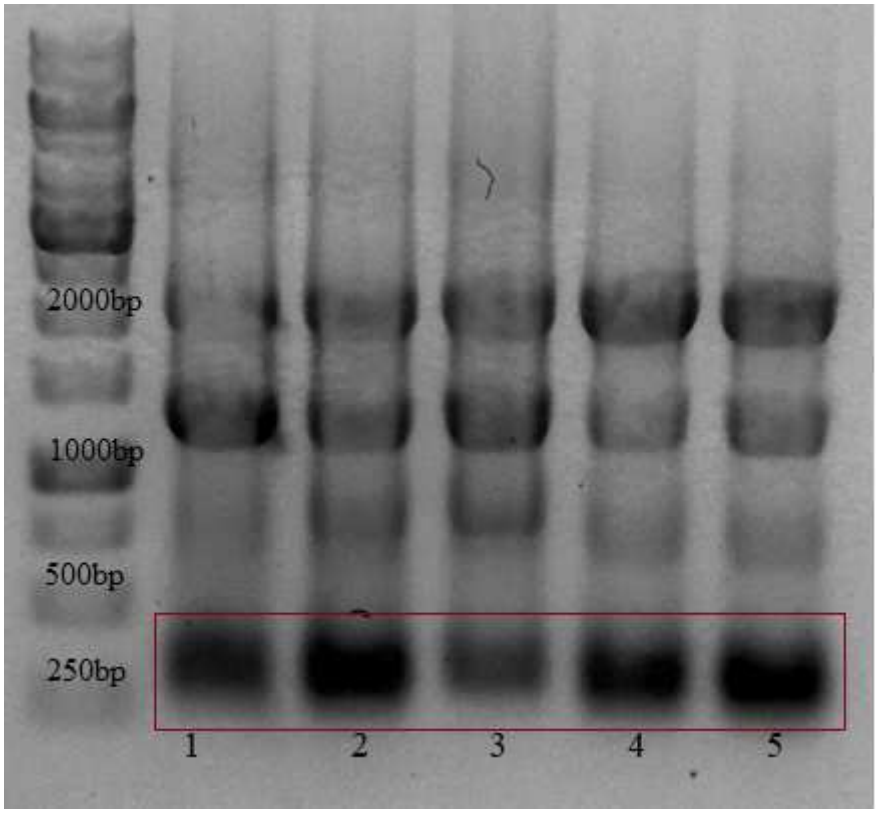


Figure 16 Colony PCR of selected clones. Lanes 1-5 are, in order, the amplifications of: clone 1, clone 2, clone 3, clone 7 and clone 8. Each well was loaded with 3µl of DNA sample.

Then the plasmid has been extracted from the most promising clones (clones 7 and 8, Figure 17) and checked though electrophoresis (Figure 18). Figure 18 shows the different supercoiling of the two extracts, eluted in water.

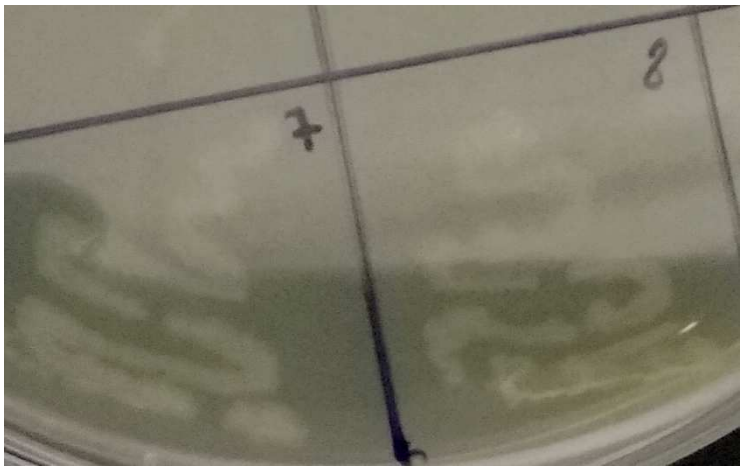


Figure 17 Subcultures of clones 7 and 8 on LB and ampicillin.

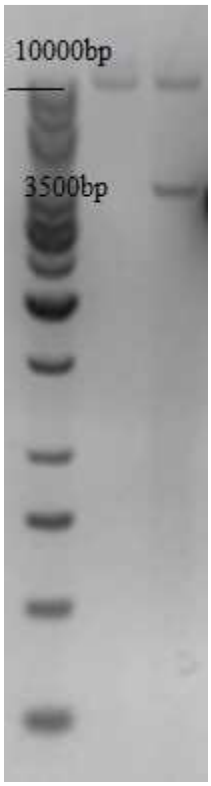


Figure 18 AlcA::bLPL plasmid extracted from *E. coli* clones 7 and 8, respectively. Each well was loaded with 3µl of DNA sample.

Extracted plasmids were quantified at the spectrofluorimeter (see table 39) and diluted 1:30 in SDS water for PCR purposes.

Table 39 Quantification of the plasmids AlcA::bLPL extracted from clone 7 and 8.

Clone	**AlcA::bLPL plasmid concentration (ng/µl)**
7	**25.92**
8	**70.589**

Amplifications of the internal, whole and promoter fragment followed as described in the methods, paragraph 4.3.1, except for the internal sequence which was amplified with a melting temperature of 57°C.

Results are showed in Figure 19.

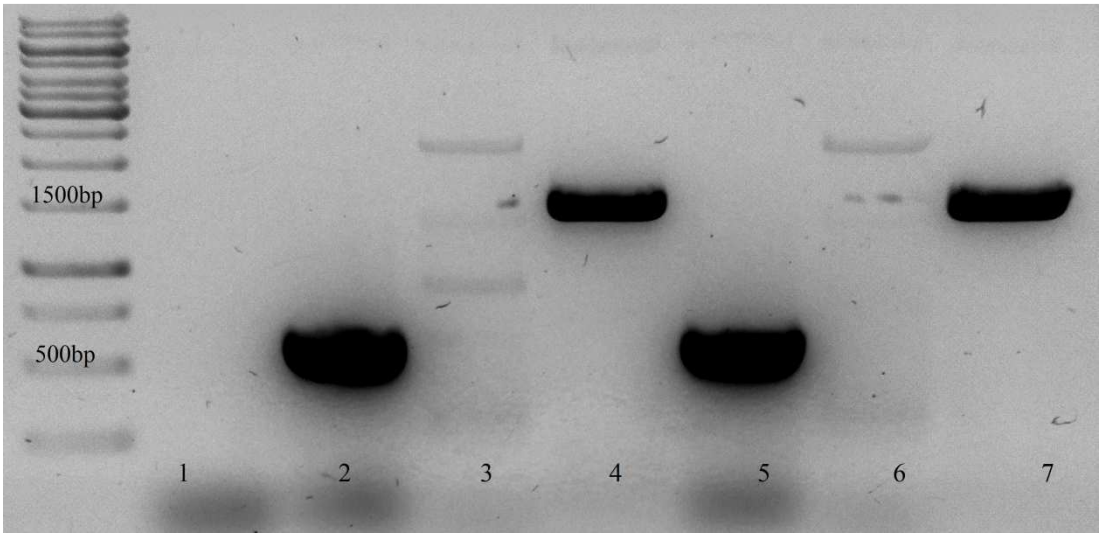


Figure 19 Molecular analyses of clones 7 and 8. In lane 1 there's the negative control: amplification of the promoter sequence in AlcA plasmid; lanes 2-4 show the amplification of the promoter, the internal and the whole bLPL sequence in AlcA::bLPL extracted from clone 7; lanes 5-7 show the amplification of the promoter, internal and whole sequence of bLPL in AlcA::bLPL extracted from clone 8. Each well was loaded with 3µl of DNA sample.

As it can be inferred in the picture above, both plasmid extracts contain the sequence of interest and its promoter. The amplification of the internal sequence gave several aspecific bands and needed optimization, which we did, until it worked well as in Figure 20, where the melting temperature was set at 60°C as described in Methods, paragraph 4.3.1.

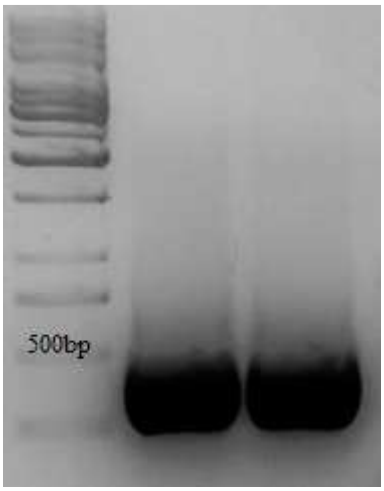


Figure 20 Amplification of the internal sequence of 451bp of the plasmid extracts from clones 7 and 8 respectively. Each well was loaded with 3µl of PCR product.

Plasmid extracts from the two clones, which were proved to be identical by the molecular analyses, were used afterwards for *Aspergillus* transformation (clone 7) and as positive controls (both).

6.2 *Aspergillus nidulans*

6.2.1 Light Microscopy

Both bacterial and fungal strains were observed at the optical microscope to check their fitness and any microscopic morphological mutation. Pictures of three of selected strains follow.

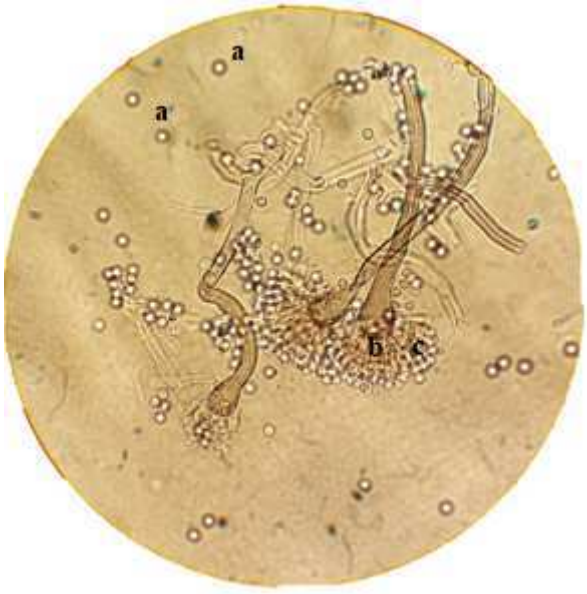


Figure 21 Transformant t18 at the optical microscope. Magnitude 100X.**a.** spores **b.** metulae **c.** phialides

Figure 22 Transformant t15 at the optical microscope. Magnitude 100X. **a.** spores **c.** phialides

As figures 21 and 22 show, both strains show a wild type morphology and appear healthy presenting lots of spores (a) and well-developed metulea (b) and phialides (c).

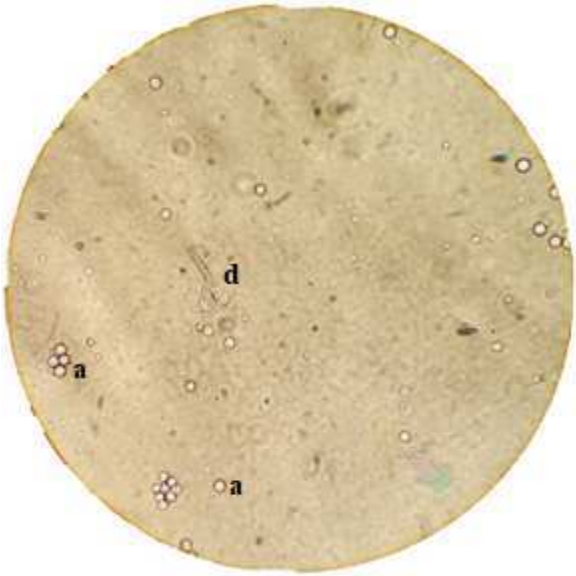


Figure 23 Transformant t8 at the optical microscope. Magnitude 100X.**a.** spores **d.** conidiophore

Transformant t8 (Figure 23), on the other hand, shows mutations to the conidiophores (d), which appear as bilobate. Even the amount of spores (a) is rather poor, suggesting few healthy conidiophores. The strain was induced, and proteomic analyses showed the production of a truncated form of the protein bLPL.

6.2.2 *Aspergillus* transformation

In order to be transformed *Aspergillus* spores need firstly to be turned into protoplasts (fungi cells deprived of their outer walls) to allow entrance to the plasmid. Protoplasts show themselves as faded or out of focus spores with a much thinner and much less blackish outer circle surrounded by a translucent glow, that is like ghost spores (see Figure 24). Being deprived of their outer wall makes these cells extremely fragile and they have to be handled accordingly (for example without ever taking them out of the ice bath). In fact, the first attempt at making protoplasts resulted in zero transformants and the morphological analysis of the white background fluff showed little and wrinkled spores that could not be captured in a photograph. These spores seemed under osmotic stress, but nutritional problems could not be excluded. Therefore, all solutions (Table 11) were made new to avoid osmotic problems, nutrients were given as individual supplements instead of adding a vitamin mix. It was also suggested to add double the amount of magnesium in a separate plate, according to Manoli and Espeso (2019) results. This time, all plates grew transformants, apparently without any difference relating to magnesium concentrations.

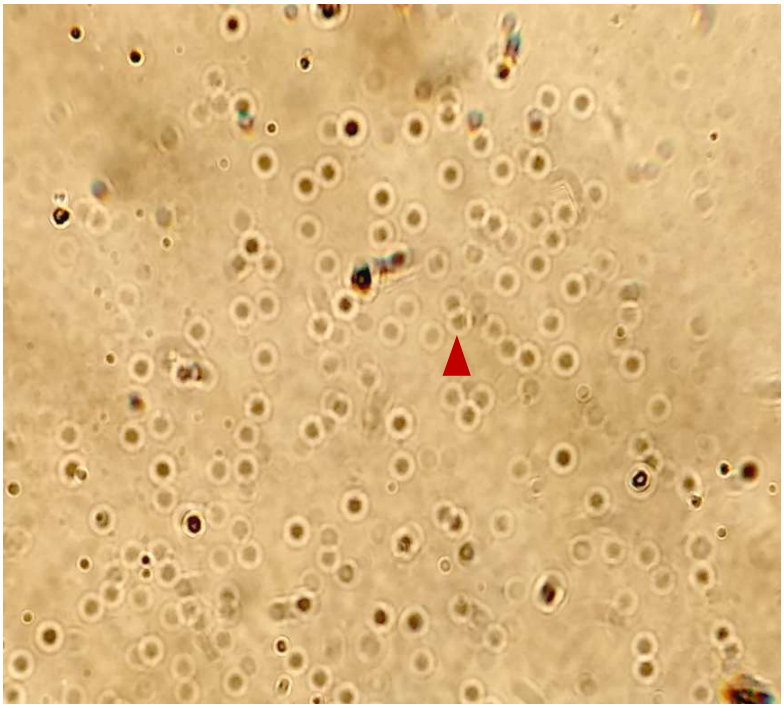


Figure 24 *Aspergillus* protoplasts (example indicated by red arrow) at the optical microscope. Magnitude 40X.

Transformed Aspergilli are thus plated on Top agar with all the supplements except arginine and incubated at 37°C for three to five days or as long as it takes transformants to appear and differentiate from the whitish background (Figure 25).

Figure 25 A Top Agar plate with transformants growing, although still not recognisable.

Thus, transformants are picked from the Top Agar plates and into Complete Medium plates (Figure 26). Transformants, in fact, are highly stressed and could recognise the inserted sequence as non-self at any moment, thus exciding and digesting it. Allowing them to grow on a richer medium should assure their fitness and a certain resistance against minor stresses like a well-integrated DNA sequence. As Figure 26 shows, on CM transformants grew radially, exhibiting different sectors.

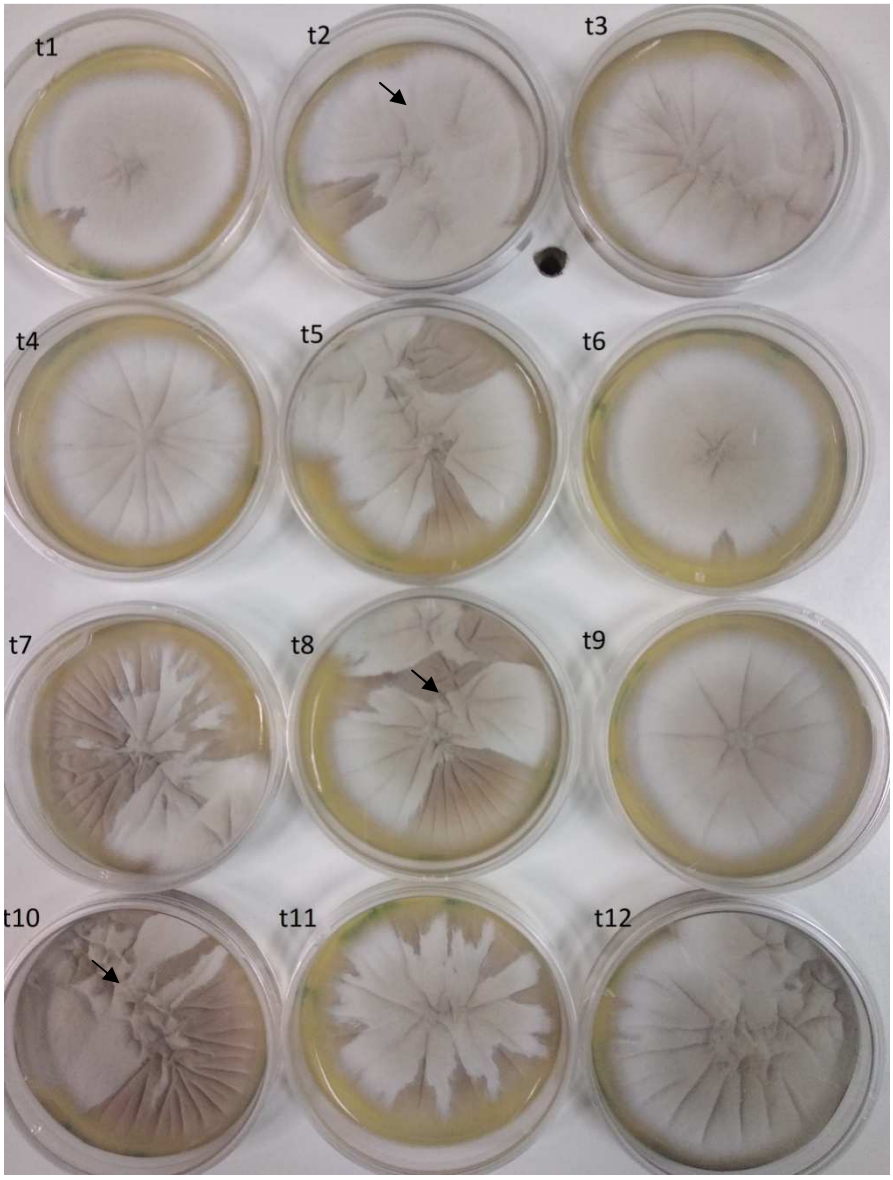


Figure 26 *Aspergillus* transformants on Complete Medium.

At this point well grown strains can be replicated on AMM, in order to make spore solutions from which the genomic DNA can be extracted.

Both cultures on CM and AMM showed liquid drops developing at the origin on numerous sectors (like in figure 6, blue arrow). These exudates may contain interesting metabolites that should be analysed.

Contrary to *E.coli*, the fungus integrates the plasmid into its own genome at the *argB* locus, where it restores its arginine auxotrophy. Transformants appearing on the medium without arginine have surely integrated the vector, but *Aspergillus* may have lost the nucleotide sequence. Molecular analyses thus followed to ascertain the presence and the entirety of the sequence of interest.

6.2.3 Transformants selection

Figure 27 shows an example of the amplification of the internal sequence of several transformants.

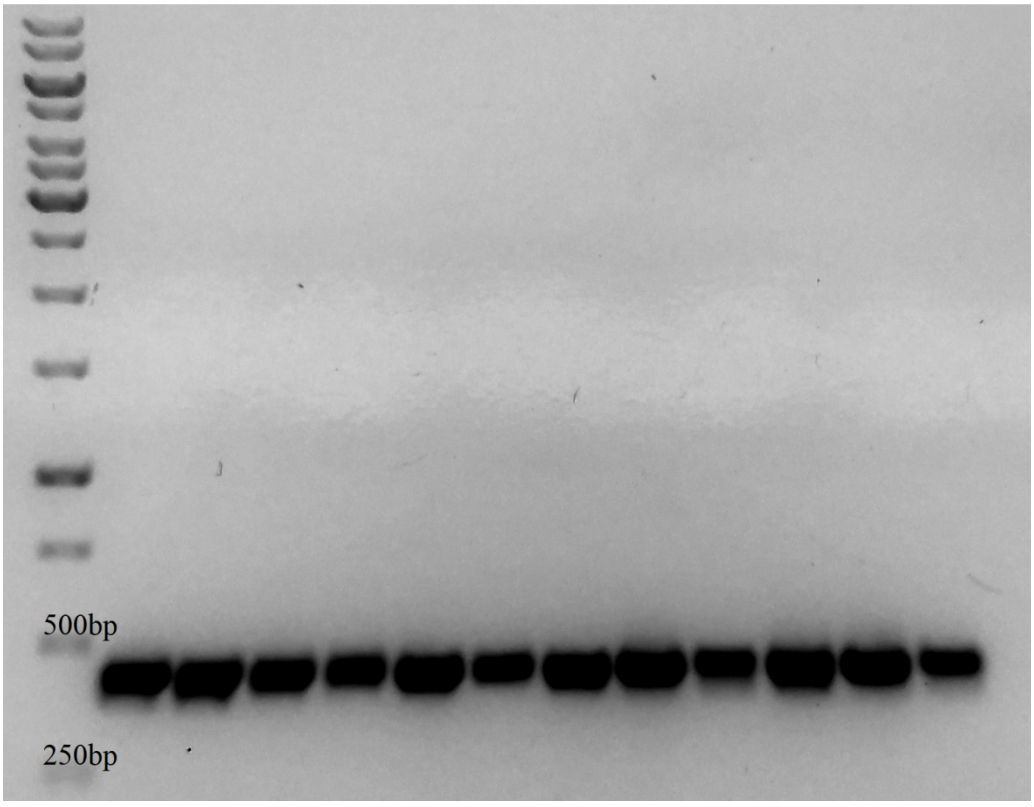


Figure 27 Amplification of the internal sequence of 451bp of transformants t1-12. 3µl of sample were added in each well.

Figure 28 shows the amplification of the whole bLPL sequence of t6.2. The band pointed with the black arrow is rather faint, which is partly due the adjacent presence of the huge light of the positive control. Moreover the amplification of the whole sequence from genomic DNA is troublesome because of the interference of the genomic DNA and also because of the tail of the reverse primer.

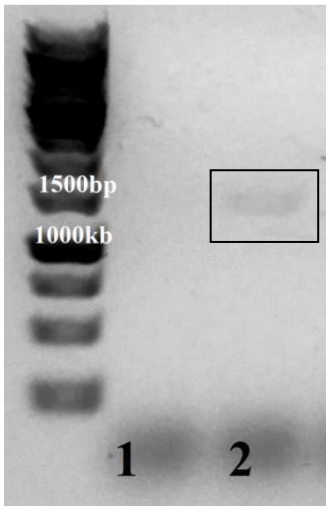


Figure 28 Amplification of the whole bLPL sequence (1462 bp) of t5 (lane 1, no amplification) and t6 (lane 2). 3µl of sample were added in each well.

Figure 29 shows the amplification of the promoter region with the primers AlcA_up_fw and bLPL_int_rv carried out with the Red Mix Polymerase.

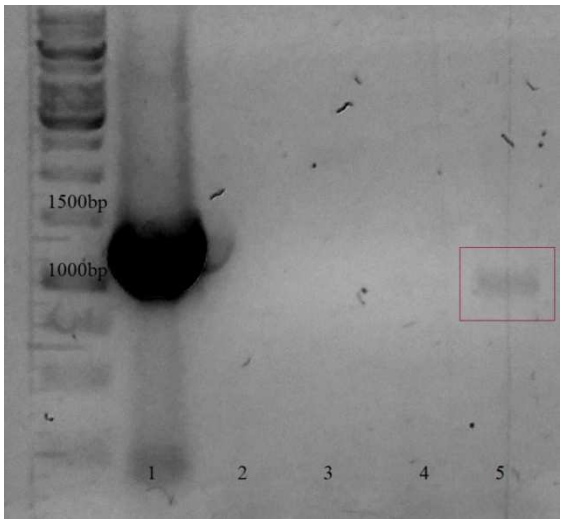


Figure 29 Amplification of the promoter region with primers AlcA_fw, bLPL_rv, 1099bp. Lane 1: positive control. Only transformant t6.2 showed amplification. 3µl of sample were added in each well.

6.2.4 Induction

Selected strains t6 and t8 were induced with ethanol and incubated for 25h, then harvested and further handled for proteome analyses (Results 6.4).

Then transformants t15 and t18 were also induced in three different conditions:

- with 1% ethanol for 25 hours;
- with glucose, then 30 minutes starvation, then 1% ethanol;
- with 1% ethanol and BSA 1% for 25 hours;

BSA was added to try and stabilize the monomeric form of the protein (Hajashi *et al.*, 1986).

A new transformation was performed and transformants t6.2 and t10.2 were also induced but only with 1%ethanol, which had already proved to be the best inducing condition for these transformants. Macroscopically, there are clear differences between SAA111 and bLPL transformants in broth AMM. Even in AlcA repressing conditions, SAA111 forms roundish vesicles and an abundant mycelium. The transformant, on the other hand, grows into a more disperse vesicles emulsion with a thinner layer of mycelia and increases the viscosity of growing medium (Figure 30). All transformants grew similarly to t6 in Figure 30.

Figure 30 Differences between SAA111 (left) and transformant t6 (right) in glucose.

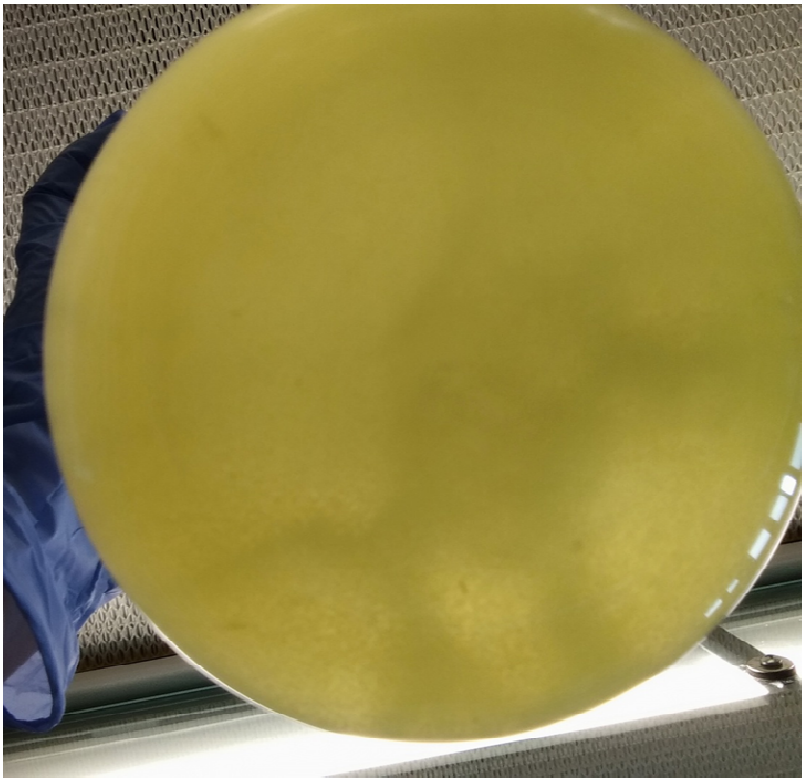


Figure 31 Induced straint18 in 1% ethanol and 1% BSA.

BSA rendered the culture even more opaque (Figure 31), the medium was the most viscous and dispersed in many vesicles.

6.3 Construction of AlcA::bLPL

AlcA plasmid and bLPL plasmid were both digested with the restriction enzymes *EcoRI* and *BamHI* (Table 16) generating sticky ends.

bLPL proved impossible to purify in the last stages, whether because it was too difficult to elute or because it eluted too early from the purification membrane. To rule out any problem with the plasmid scaffold itself, a restriction sites analysis has been performed with *EcoRI, BamHI, XhoI, HindIII* and a coupled restriction with *XhoI* and *HindIII* that can both be used with the TANGO 1X or 2X buffer (Figure 32). As it can be observed in Figure 32, all restriction enzymes cut in one site and the *HindIII*/*XhoI* coupled restriction gave a band corresponding to the open plasmid, but with a lower molecular weight and a band weighting a little more than 1500bp corresponding exactly to the manufacturer's scheme of the scaffold.

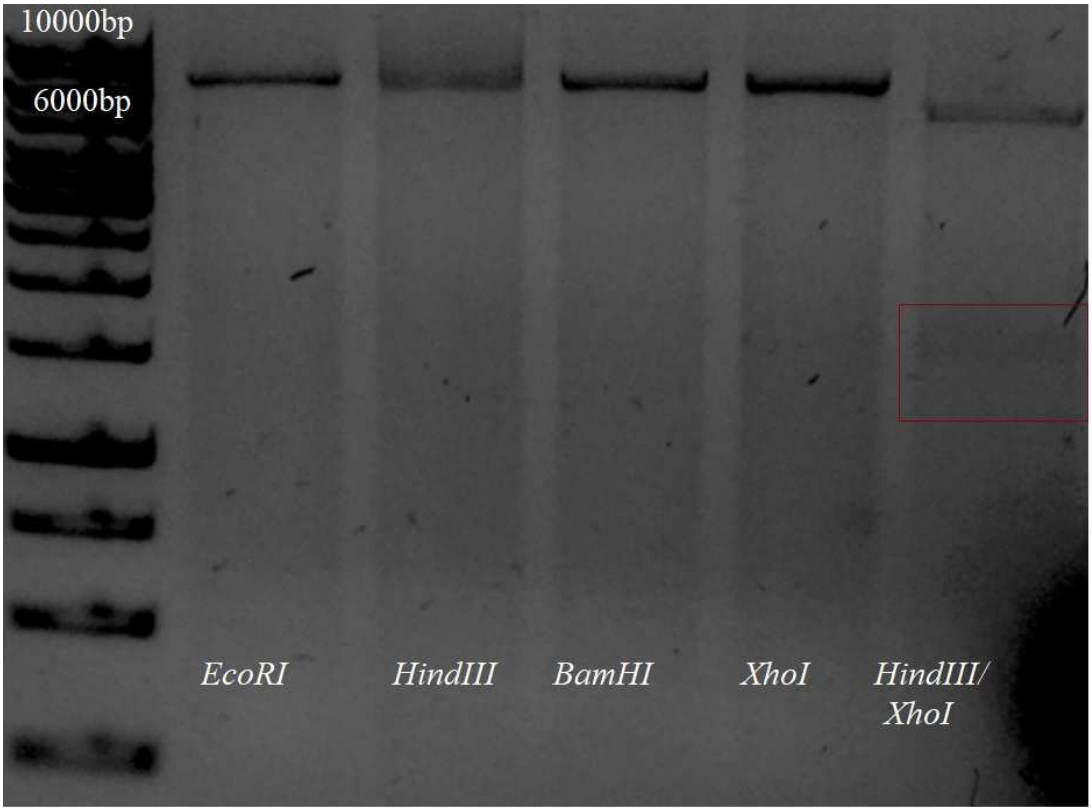


Figure 32 bLPL plasmid restriction patterns. The band in the red box, hardly distinguishable, in the picture is the fragment excised by *HindIII* and *XhoI*. 5µl of sample where loaded in each well.

The desired bLPL sequence was then achieved by amplifying it by PCR with the specific primers bLPL_BamHI_fw and bLPL_Cstrep_EcoRI_rv: the resulting construct has the Strep-tag® at the C-terminal end for purification and/or western blotting purposes. The amplified sequence has been digested with *BamHI* and *EcoRI* as already described and quantified just like AlcA plasmid (Figure 33) and then ligation between the restricted AlcA and bLPL-Cstrep followed by means of the T4ligase and its buffer incubated at 4°C overnight. A graphical representation of the achieved construct is presented in figure 34.

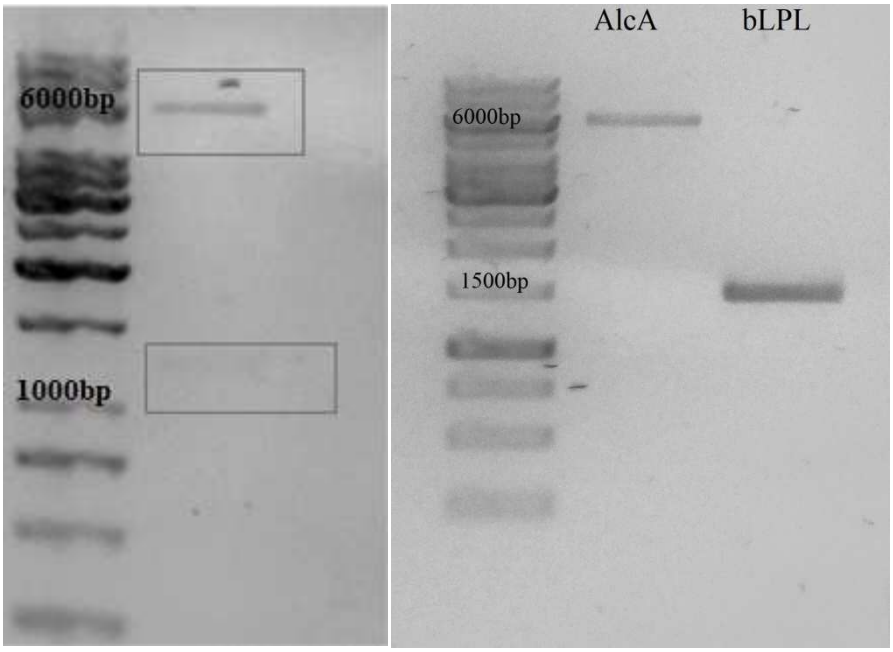


Figure 33 AlcA digested with *EcoRI* and *BamHI* (left) and AlcA and bLPL before ligation (right). 5µl of sample were loaded in each well.

Figure 34 Open graphical representation of the constructed AlcA::bLPL plasmid.

6.3.1 gLPL

At the beginning of this work of thesis, LPL from *Gallus gallus* was also taken into consideration. The sequence embedded in a scaffold just like the bLPL was bought and handled exactly like the bovine one. The plasmid was digested with the enzyme

EcoRI and subsequently with *BamHI* highlighting at least two restriction sites for the latter (Figure 35).

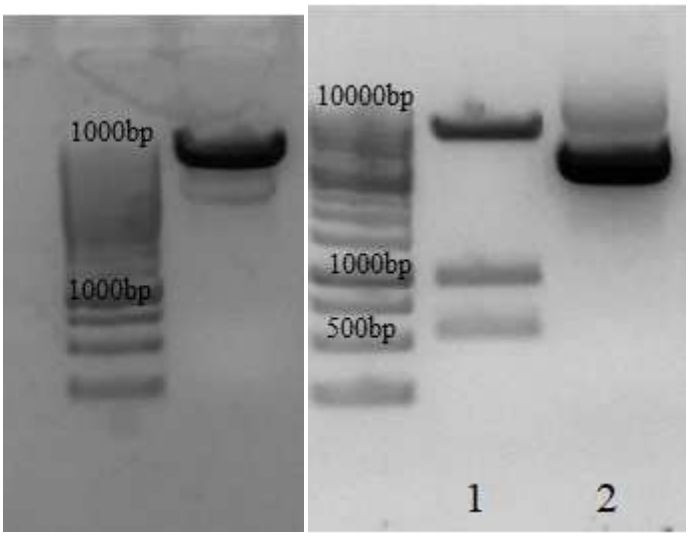


Figure 35 gLPL digested with *EcoRI* (left) and gLPL further digested with *BamHI* (lane 1) and as it is after extraction from *E. coli* in lane 2 (right). 5µl of sample were added in each well.

It wasn't possible to amplify the sequence with the same primers as the bLPL one (Figure 36), thus it was put on hold. However, the same restriction analysis as for bLPL was performed to appreciate at least a qualitative comparison between the two (Figure 37).

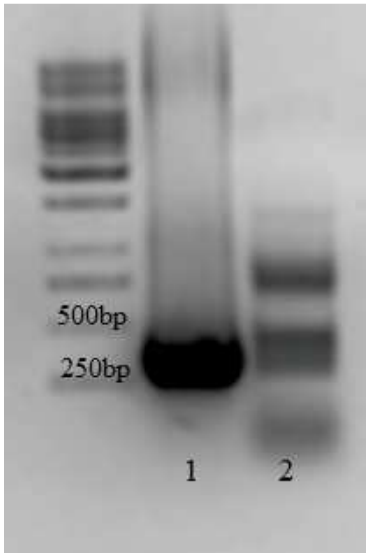


Figure 36 bLPL (lane 1) and gLPL (lane 2) amplified from the respective plasmids with the bovine internal primers. 3µl of sample were loaded in each well.

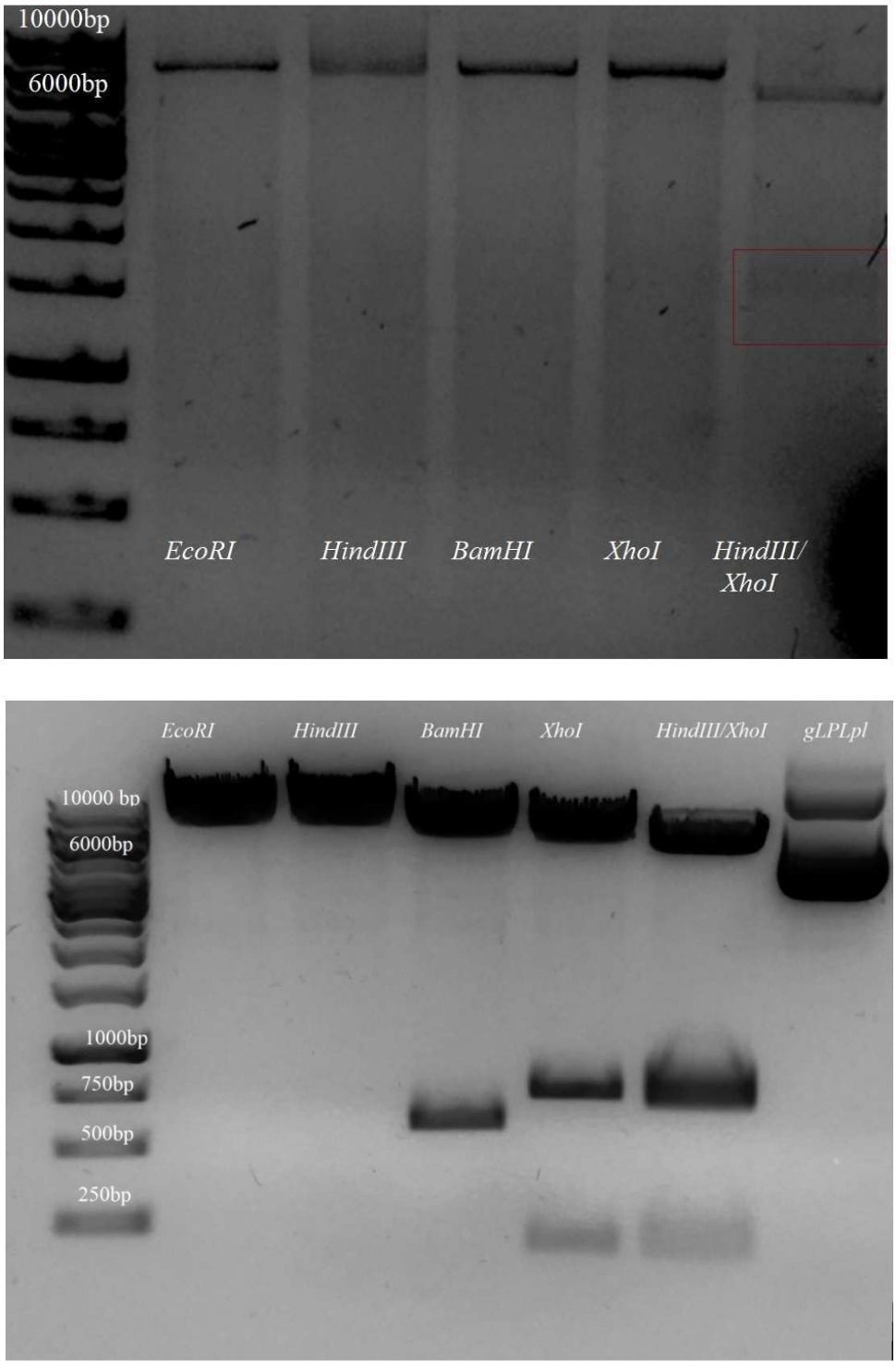


Figure 37 Restriction profile of bLPL (up) compared to the restriction profile of gLPL (down, last lane is gLPL plasmid). 5µl of sample were added in each well.

It can be noted that all of the utilized enzymes except for *EcoRI* and *HindIII* find more than one restriction site on gLPL plasmid

6.4 Proteomics

6.4.1 Bradford

Figure 38 shows the BSA standardization curve described in 4.4.2.1.

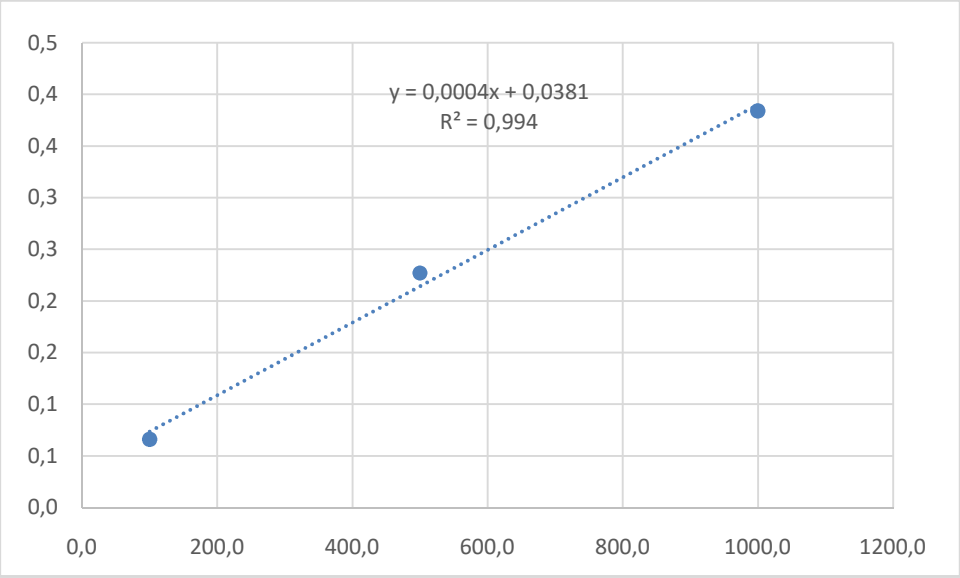


Figure 38 BSA standardization curve.

6.4.2 Sample quantification

Here is just one example of sample quantification and volume measurements as it was carried out throughout the present thesis work. Protein samples were titrated to 20µg in a maximum volume of 40µl for the bigger combs and 20 µl for the smaller ones except when their concentration was estimated too low for the total volume.

Table 40 Proteic samples titration calculations.

$Abs_{\backslash 280nm}$	Total µg	Sample volume	4XSample Buffer volume	MilliQ water volume
0.786	1969.75	10.7	5	4.3

6.4.3 Coomassie and Western blotting

Purification with the Heparine-Sepharose column gave no useful results, while the use of the Strep-Tactin® one proved better. Figure 39 shows the Coomassie of the last purification. In lane 1 there's the crude extract of transformant t6 induced with 1% ethanol for 25 hours. In lanes 2 to 7 there are: the column flow through, the first washing, that last washing, fractions 1 and 2. In lanes 8, 9 and 10 there are the fractions 3, 4 and 5 where it can be appreciated at least one main band (although there were several).

Figure 40 shows the western blotting performed on a gel which had run in parallel to the previous Coomassie, showing two bands recognised by the anti-Strep antibody in the last three fractions corresponding to 135kDa and 75kDa. The main 75kDa band is the bovine *lipoprotein lipase*, while the other one may be an aggregated form.

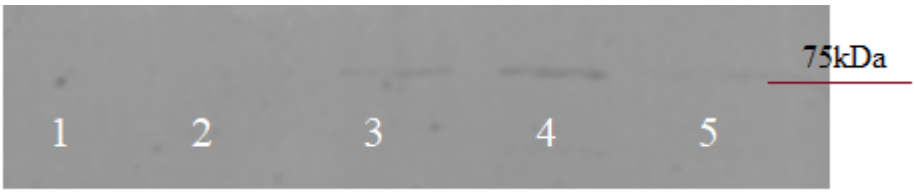


Figure 39 Coomassie staining. Purification performed with the Strep-Tactin® column. Lane 1: final washing; lane 2: fraction 1; lane 3: fraction 2; lane 4: fraction 3; lane 5: fraction 5. Washing and fractions were not titrated. (Full picture in Appendix 2)

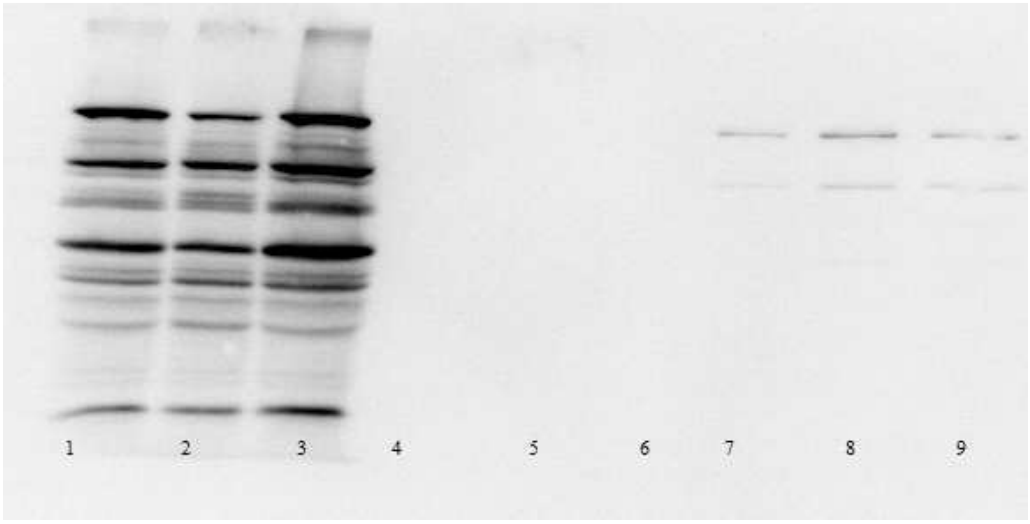


Figure 40 Western blotting with anti-Strep antibody. Purification performed with the Strep-Tactin® column. Lane 1: crude extract of t6 induced for 25h; lane 2: flow through; lane 3 washing 1; lane 4: washing 12; lane 5; fraction 1; lane 6: fraction 2; lane 7 fraction 3; lane 8: fraction 4; lane 9: fraction 5.Crude extracts were titrated to 20µl; washing and fractions were not titrated.

It seems worth mentioning the first expression attempts.

Firstly, two transformants were induced with ethanol and showed two interesting bands at 35kDa and more than 100kDa, which may be the truncated and aggregated form of the bLPL (Figure 41).

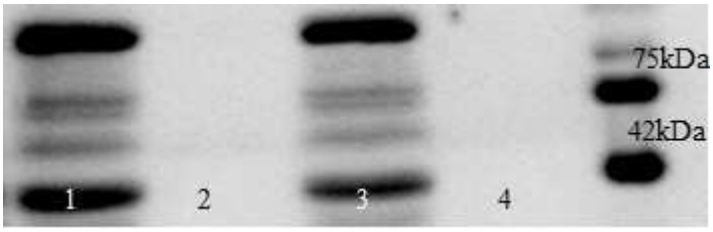


Figure 41 Western blotting with anti-strep antibody of transformants t6 and t8. Lane 1: crude extract of t6; lane 2: supernatant of t6; lane 3: crude extract of t8; lane 4: supernatant of t8. Crude extracts samples were titrated to 20µg, while supernatants were not titrated. (Full picture in Appendix 2)

The other interesting expression trial was with addition of BSA. The BSA band at 66kDa was over expressed (white in figure 42, lanes 1 and 2). However, treatment of the membrane with the anti-strep antibody showed two bands just under it that might correspond to the bLPL(Figure 42, black and blue arrows),ranging from 45 to 66kDa

of MW and being recognised by the anti-strep antibody as discussed in chapter 7. The marker 66kDa band showed in the Coomassie is itself BSA.

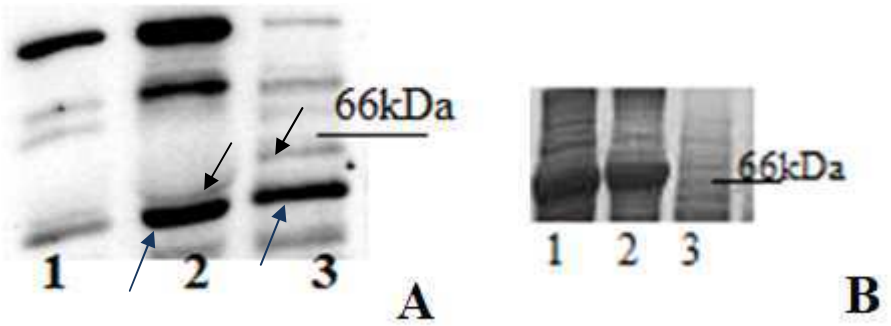


Figure 42 Western blotting with anti-strep (A) and relative Coomassie (B) of the crude extracts of SAA111 with 1% of BSA (lane 1), induced t15 with 1%BSA (lane 2) and induced t15 without BSA (lane 3) . Samples were titrated to 20µg. (Full picture in Appendix 2)

7. DISCUSSION

The bovine *lipoprotein lipase* found in milk has been widely investigated in the past in terms of its physical and chemical characteristics and purification methods, but its function and role in milk is still poorly understood (see Paragraph 1.1). In the aim of this research, a new system to produce pure bLPL was tested.

In order to produce the construct for the recombinant production of bLPL in *Aspergillus nidulans*, a restriction assay was firstly performed to ascertain the presence of bLPL in the purchased plasmid (Figure 32). The experiment consisted in the digestion of bLPL plasmid with *EcoRI* and *BamHI*. Thus, the method required digestion with one restriction enzyme, purification from the agarose gel, subsequent digestion with the other restriction enzyme and once again, excision and purification of the digested sequence of interest from the agarose gel. This method proved to be quite difficult, since the sequence was often lost in the last step for technical problems.

Subsequently, the bLPL sequence was amplified with forward and reverse primers containing the restriction sites *BamHI* and *EcoRI*, respectively. The reverse primer also includes the sequence encoding for eight amino acids peptide, the Strep-tag® (Table 15). The restriction with *EcoRI* and *BamHI* was still necessary to achieve sticky ends that would allow the sequence to combine with the vector AlcA (Figures 33 and 34). The Strep-tag® allows purification with the Strep-Tactin® as well as recognition by the anti-strep antibody. After a little adjustment of amplification parameters with the Phusion® Master Mix polymerase, we achieved the amplicon of interest and proceeded digesting it. The AlcA plasmid, extracted from *E. coli*, was also digested with the same restriction enzymes. After quantification and observation of the two sequences on the agarose gel, ligation by means of T4 ligase was performed. After few trials, the best plasmid-insert combination was achieved by mixing AlcA and bLPL in the ratio 1:3 and incubating for 2 hours at room temperature (Figure 33, right). The resulting plasmid, AlcA::bLPL-Cstrep is the vector chosen for the new system.

Competent *Escherichia coli* DH5α cells were thawed, plated and grown on LB medium (Table 3). Replication and growth allow cells to lose their competence. A fresh batch of chemically competent E. coli cells was generated to be transformed with the ligation mix containing the AlcA vector, specific for *A. nidulans*, and the bLPL cDNA. Competent cells were then assayed to test their efficiency.

Competent DH5αs were transformed efficiently with the selected vector, colonies appeared after an overnight incubation at 37°C and clones could be isolated on a master plate. All clones grew and a rough colony PCR was performed on some selected ones.

Two clones were chosen and analysed for the presence of the vector and its integrity as described in Methods 4.3.

The plasmid was run on agarose gel to get its fingerprint and PCR mixes and cycles were optimised when needed.

In the end, both of the chosen clones had the desired vector in all its components.

At this point *Aspergillus nidulans* pre-cultures on plates were harvested and turned into protoplasts by enzymatic digestion of the outer fungal wall and mechanical separation of the protoplasts, which were immediately transformed with the vector extracted from one *E. coli* clone. The generation of protoplasts is a delicate procedure in which the operator can make mistakes and the fungus could go into cell death or could not internalize the exogenous DNA, resulting in the absence of transformants (see Results 6.2.3). After three transformation trials, a total of 36 transformants grew.

Genetic stabilization was achieved by replicating the transformants on complete medium (Figure 26) and then again on minimal medium as described in the methods.

If the method is successful *Aspergillus* integrates the desired sequence into its genome at the *argB* site starting from the *AlcA* promoter at 5’ to the *trpC* terminator at 3’. *Aspergillus* could recognise the exogenous sequence at any time and excise it,

even keeping the arginine auxotrophy-restoring sequence. This is another risky step in the genetic manipulation of *Aspergillus*.

All grown strains were analysed for the presence of the internal sequence of the bLPL. Positive transformants were then probed for the whole sequence and then for the promoter sequence. All PCR cycles were adjusted for amplification from genomic DNA.

Selected strains were grown in broth to be induced with different carbon sources to find the best one. They were grown with glucose, to have a negative control and investigate any basal production levels; with glucose overnight, followed with half an hour of starvation to eliminate any glucose residues within the cell and then with 1% of ethanol, which led to high amounts of biomass but no bLPL production. Lastly, they were directly induced with 1% of ethanol for 25 hours, obtaining the best results.

After induction the biomass was harvested and collected by means of membrane filtration and immediate freezing in liquid nitrogen to prevent *Aspergillus* from activating any stress response against osmolar stress thus hyper producing proteases. All transformants were observed at the optical microscope in search of any morphological change. The first two induced transformants showed significant morphological changes (Figure 23).

Proteomic analyses showed the expressed protein in the crude extract, rather than in the growing medium. Since bLPL in milk is mainly embedded in casein micelles, it may be reasonable to think that the protein needs a stabilizing vector to physiologically get outside of the cell. Therefore, it could be useful to add a signal peptide in the recombinant *Aspergillus* to lead bLPL directly in the medium and detect a possible stabilizer, specific for bLPL.

The proteomic analyses on crude extracts of the first induced transformants, showed what may be a truncated form of the protein around 35kDa and one around 100kDa (Figure 41) that may be the aggregated form of the protein or the homodimer, as other authors have observed (Kinnunen *et al.*, 1976).

Micellar interactions could help bLPL stability in milk, where the anchor GPIHBP1 and heparin are absent. In order to avoid aggregation1% BSA was added as it was reported that BSA stabilises the enzyme activity and thus its conformation (Hajashi *et al.*, 1986). BSA however introduced a new level of difficulty: it weights 66kDa and juxtaposes with the bLPL (Figure 42). At first the Coomassie staining and the Western Blotting with the anti-LPL antibody seemed to prove that there was no LPL, but further recognition with the anti-strep antibody made it seem like the protein was only covered by the BSA, giving rise to the question whether the latter can also somehow react with the bLPL in a way as to cover the anti-LPL antibody epitope. The anti-LPL antibody, however, showed less specificity than the anti-strep one throughout the whole work, therefore the absence of recognised bands in the membrane (not showed here) could be solved by using a different anti-LPL antibody. In Figure 42, the bands highlighted with black and blue arrows are at 66kDa and between 45kDa and 66kDa respectively, are recognised by the specific antibody and could represent bLPL at two glycosylation degrees. In particular, the band pointed with the blue arrow in lane 2 is way more intense, suggesting stabilization has occurred with the BSA together. Upon further induction with the same transformants the protein was not expressed. Molecular checks on the strains were once again performed and the exogenous DNA was found to be absent in the new t15 generation. Further analyses are needed to confirm the arrow-pointed bands in Figure 42 are different forms of the bLPL: activity assays, protein sequencing, trials with the new selected transformant t6.2 and repeating the induction with transformant t15 by thawing its first generation stored at -80°C.

A new transformant was finally selected and proved positive for the integration of the whole sequence of interest, AlcA::bLPL-Cstrep (expression and purification results of this transformant are showed in Figures 39 and 40).Purification was attempted with all samples showing the presence of LPL. The following methods were attempted:

- low salt extraction and purification with the Heparin-Sepharose column with Tris-based solutions;

- low salt extraction and purification with the Heparin-Sepharose column with PBS-based solutions;
- low salt extraction and purification with the Strep-Tactin® column;
- high salt extraction and purification with the Strep-Tactin® column.

In literature the bovine LPL is purified from milk by ultracentrifugation or combined steps of column chromatography with the Heparine-Sepharose column starting from high volumes of milk (10-40L) (Tajima *et al.*, 1984; Jensen and Pitas, 1976; Hayashi *et al.*, 1986). This thesis deals with an experimental system much smaller, with crude extracts volumes ranging from 500µl to 5ml. Moreover, the matrix hosting bLPL is completely different from the milk "tissue". For these reasons different purification procedures were tried. The lack of positive results with the heparin-sepharose column may be due to some component of the crude extract matrix covering the heparin-binding site, to aggregation or to the wrong folding of the protein in the ER, which is the most probable reason without its chaperone, LMF1.

Significant results were achieved only with the last inducing condition with transformant t6.2, which showed two antibody-recognised bands at 135 and 75kDa (Figure 40). Given the broad MW spectrum literature attributes to bLPL as described in 1.1.3, the 75kDa band could be bLPL with a high degree of glycosylation, while the 135kDa one could be the protein aggregate. Literature shows different forms of bLPL between 42 and 66kDa (see Introduction). Forms of 75kDa could be the result of recombinant expression in *Aspergillus*, which may have higher glycosylation or nitrosylation rates than animal cells. The purification of the 135kDa form may imply difficulties in the aggregate form purification and it confirms the need to stabilise the monomeric form of the bLPL whether by co-expressing its chaperone or chemically adding stabilising molecules like BSA or heparin.

The purified protein (Figure 39) could not be quantified with the Bradford assay. The spectrophotometer could read its absorbance, but it was slightly less than the first point in the standard curve, therefore its concentration is less than 0.1µg/ml (see Materials 3.4.2). However, it could be seen in the Coomassie stained membrane (Figure 39), the sensitivity limit of which is 100ng or slightly less. Therefore, the

purified protein is reasonably quantifiable around 50ng/ml. By considering that some of the protein could be lost in the purification procedure, bLPL is produced in *A. nidulans* even if in low amounts. This happens because *A. nidulans* is a model organism not utilized for high-rate protein production but to test the possibility of producing a specific protein in *Aspergillus* genus.

8. CONCLUSIONS

This work established a new system for the production of bLPL.

The plasmid AlcA::bLPL was constructed and used to transform *Aspergillus nidulans*. The new cell factory proved able to express the bovine *lipoprotein lipase* inside the cytoplasm. Proteomic analyses confirmed its presence only in the crude extracts (Figure 41) and it could be useful therefore to add a signal peptide that leads the protein in the medium.

BSA was added to the medium during induction in an attempt to stabile the active form of the enzyme. Proteomic results (Figure 42) were interesting suggesting BSA could actually be useful in stabilising bLPL. Further analyses to confirm BSA stabilising effect and to minimize its background noise are required. Moreover, trials of *Aspergillus* cultures with BSA are not available and it's not known whether the fungus allows entrance to the protein and metabolises it, leaves it like it is in the growing medium, digests it in the medium and then incorporates the amino acids. Macroscopical observations in this work suggest that at least BSA changes the vesicular production of *Aspergillus* (Figure 31) and its proteomic rational outcome (Figure 42). It may be interesting to investigate whether *Aspergillus* produces new secondary metabolites and how it actually behaves upon BSA trigger. Specifically, it should be investigated the molecular pathway of *Aspergillus* bLPL production and how it shifts when adding BSA. In the short term, the analyses suggested in Discussion could confirm BSA contribution to bLPL stability.

Transformants induction conditions should be further explored, using different times and temperatures, modulating magnesium and calcium concentrations (Manoli and Espeso, 2019) and adding different AlcA-inducing carbon sources like fructose or 2-butanone.

Moreover, it is useful to remember that LPL needs a chaperone protein to fold properly and co-expression of the latter with the AlcA::bLPL-Cstrep should be a mandatory step towards a fully functioning recombinant expression system.

Nonetheless, bLPL was purified from a selected transformant, using the Strep-Tactin® system, even if in amounts that could not be quantified with the Bradford assay directly but it's estimated to be around 50ng/ml.

The purification steps also need optimization and it may be worthwhile trying optimising the heparin-sepharose column once again since it is the one traditionally used in literature (Tajima *et al.*, 1984; Jensen and Pitas, 1976; Hayashi *et al.*, 1986). Also ultracentrifugation is suggested, whether possible. Activity assays should follow to further test bLPL identity and correct folding.

The work presented in this thesis represents the starting point for the development and optimization of cell factories for the production of bLPL based on *Aspergillus* genera.

APPENDIX 1

Abbreviations

A. flavus – Aspergillus flavus

A. fumigatus– Aspergillus fumigatus

A. nidulans - Aspergillus nidulans

A. oryzae- Aspergillus oryzaeù

A. parasiticus- Aspergillus parasiticus

A. terreus- Aspergillus terreus

AMM – *Aspergillus* Minimal Medium

ApoC2 - Apolipoprotein 2

Arg- Arginine

Asp- Aspartic Acid

bLPL- bovine *Lipoprotein Lipase*

BSA- Bovine Serum Albumin

CM- Complete Medium

DNA – Deoxyribonucleic acid

E. coli – Escherichia coli

EC – Enzyme Commission

ER- Endoplasmatic Reticulum

FCS - Familial Chylomicronemia Syndrome

FFA- Free Fatty Acid

fw- forward

gLPL- chicken *Lipoprotein Lipase*

GRAS – Generally Recognised as Safe

His- Histidine

hLPL- human *Lipoprotein Lipase*

IDL-Intermediate Density Lipoprotein

int- internal

LB- Luria-Bertani (medium)

LMF1- Lipase Maturation Factor 1

LPL - *Lipoprotein Lipase*

mRNA- messenger ribonucleic acid

PBS – Phosphate Buffered Saline

PCR - Polimerase Chain Reaction

pH = -Log[H_3O^+]; a measure of ionic strength

PIC- protease inhibitor complex

RCL- *Rhizophus chinensis Lipase*

rv- reverse

SDS- PAGE- Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

SDW- Sterile Distilled Water

Ser- Serine

SOC - Super Optimal Broth with Catabolite Repression

T - Temperature

T_m – melting temperature

TG - Triglycerides

T-TBS – Tween – Tris Buffered Saline

VLDL- Very Low Density Lipoprotein

Units of measure used:

Temperature

°C- Celsius degrees

Turbidity

Abs- Absorbance

OD- Optical Density

DNA length

bp- base pair

kb-kilobases

Mb- Megabases

Electric current

eV- electron volt

Mass

g- grams

mg- milligrams

Molecular mass

kDa- kilo Dalton

Time

h- hours

mins- minutes

Volume

L- Liter

ml- milliliter

µl – microliter

Concentration

M - molar

mM –millimolar

µM- micromolar

Distance

λ - wavelenght

nm-nanometer

Rate

rpm - rotations per minute

Figures:

Figure 1 Lactation mechanism in bovines. c) structure of a single alveolus showing the arrangement of the mammary gland cells and (hollow) lumen area into which milk is secreted, (d) a scheme of the mammary cell during lactation illustrating the mechanisms of protein and fats secretion and showing the principal membrane

Figure 16 Colony PCR of selected clones. Lanes 1-5 are, in order, the amplifications of: clone 1, clone 2, clone 3, clone 7 and clone 8.

Figure 17 Subcultures of clones 7 and 8 on LB and ampicillin.

Figure 18 AlcA::bLPL plasmid extracted from E. *coli* clones 7 and 8, respectively. Each well was loaded with 3µl of DNA sample.

Figure 19 Molecular analyses of clones 7 and 8. In lane 1 there's the negative control: amplification of the promoter sequence in AlcA pl; lanes 2-4 show the amplification of the promoter, the internal and the whole bLPL sequence in AlcA::bLPL extracted from clone 7; lanes 5-7 show the amplification of the promoter, internal and whole sequence of bLPL in AlcA::bLPL extracted from clone 8. Each well was loaded with 3µl of DNA sample.

Figure 20 Amplification of the internal sequence of 451bp of the plasmid extracts from clones 7 and 8 respectively. Each well was loaded with 3µl of DNA sample.

Figure 21 Transformant18 at the optical microscope. Magnitude 100X.**a.** spores **b.** metulae **c.** phialides

Figure 22 Transformant t15 at the optical microscope. Magnitude 100X. **a.** spores **c.** phialides

Figure 23 Transformant t8 at the optical microscope. Magnitude 100X.**a.** spores **d.** conidiophore

Figure 24 *Aspergillus* protoplasts at the optical microscope. Magnitude 40X. The red arrow points one of the "ghost spores" that is actually a protoplast.

Figure 25 A Top Agar plate with transformants growing, although still not recognisable.

Figure 26 *Aspergillus* transformants on Complete Medium.

Figure 27 Amplification of the internal sequence of 451bp of transformants t1-12.3µl of sample were added in each well.

Figure 28 Amplification of the whole bLPL sequence (1462 bp) of t5(lane 1, no amplification) and t6 (lane 2). 3µl of sample were added in each well.

Figure 29 Amplification of the promoter region with primers AlcA_fw, bLPL_rv, 1099bp. Lane 1: positive control. Only transformant t6.2 showed amplification. 3µl of sample were added in each well.

Figure 30 Differences between SAA111 (left) and transformant t6 (right) in glucose.

Figure 31 Induced straint18 in 1% ethanol and 1% BSA.

Figure 32 bLPL plasmid restriction patterns. The band in the red box, hardly distinguishable, in the picture is the fragment excised by *HindIII* and *XhoI*.5µl of sample where loaded in each well.

Figure 33 AlcA digested with *EcoRI* and *BamHI* (left) and AlcA and bLPL before ligation (right).5µl of sample where loaded in each well.

Figure 34 Open graphical representation of the constructed AlcA::bLPL plasmid.

Figure 35 gLPL digested with *EcoRI* (left) and gLPL further digested with *BamHI* (lane 1) and as it is after extraction from *E. coli* in lane 2 (right).5µl of sample where loaded in each well.

Figure 36 bLPL (lane 1) and gLPL (lane 2) amplified from the respective plasmids with the bovine internal primers. 3µl of sample were added in each well.

Figure 37 Restriction profile of bLPL (up) compared to the restriction profile of gLPL (down, last lane is gLPL plasmid).5µl of sample where loaded in each well.

Figure 38 BSA standardization curve.

Figure 39 Coomassie staining. Purification performed with the Strep-tactin® column. Lane 1: final washing; lane 2: fraction 1; lane 3: fraction 2; lane 4: fraction 3; lane 5: fraction 5. Washing and fractions were not titrated. (Full picture in Appendix 2)

Figure 40 Western blotting with anti-Strep antibody. Purification performed with the Strep-tactin® column. Lane 1: crude extract of t6 induced for 25hrs; lane 2: flow through; lane 3 washing 1; lane 4: washing 12; lane 5; fraction 1; lane 6: fraction 2; lane 7 fraction 3; lane 8: fraction 4; lane 9: fraction 5. Crude extracts were titrated to 20µl; washing and fractions were not titrated.

Figure 41 Western blotting with anti-strep antibody of transformants t6 and t8. Lane 1: crude extract of t6; lane 2: supernatant of t6; lane 3: crude extract of t8; lane 4: supernatant of t8. Crude extracts samples were titrated to 20µg, while supernatants were not titrated.(Full picture in Appendix 2)

Figure 42 Western blotting with anti-strep (A) and relative Coomassie (B) of the crude extracts of SAA111 with 1% of BSA (lane 1), induced t15 with 1%BSA (lane 2) and induced t15 without BSA (lane 3) . Samples should have been titrated to 20µg. (Full picture in Appendix 2)

Tables

Table 1 Strains utilized in this work.

Table 2 Vectors utilized for bLPL expression (AlcA plasmid) and as bLPL sequence source (bLPL plasmid).

Table 3 Luria-Bertani Medium

Table 4 AMM composition

Table 5 Salts solution for *Aspergillus* growth

Table 6 Trace elements added to the culture medium for *Aspergillus* growth

Table 7 Complete Medium composition

Table 8 Vitamin mix composition for complete medium.

Table 9 Top agar used to grow transformants.

Table 10 Supplements added to the AMM.

APPENDIX 2

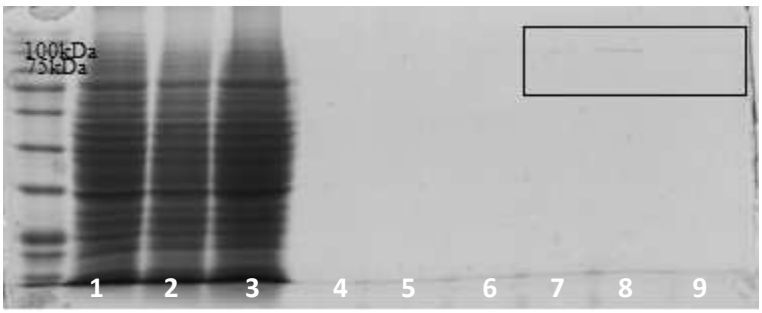


Figure 39 Coomassie staining. Purification performed with the Strep-tactin® column. Lane 1: crude extract of t6 induced for 25hrs; lane 2: flow through; lane 3 washing 1; lane 4: washing 12; lane 5; fraction 1; lane 6: fraction 2; lane 7 (hardly distinguishable in the picture) fraction 3; lane 8: fraction 4; lane 9: fraction 5. Crude extracts were titrated to 20µg; washing and fractions were not titrated but the same volume of protein suspension was loaded.

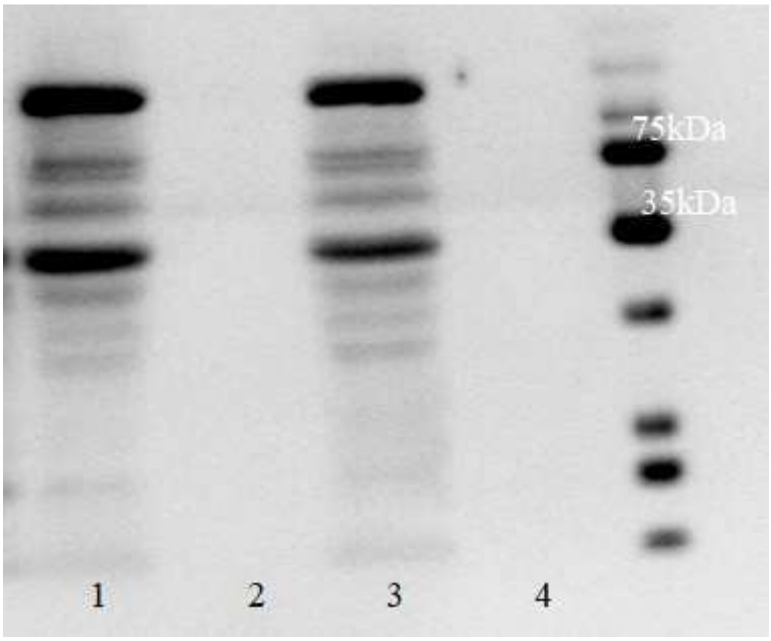
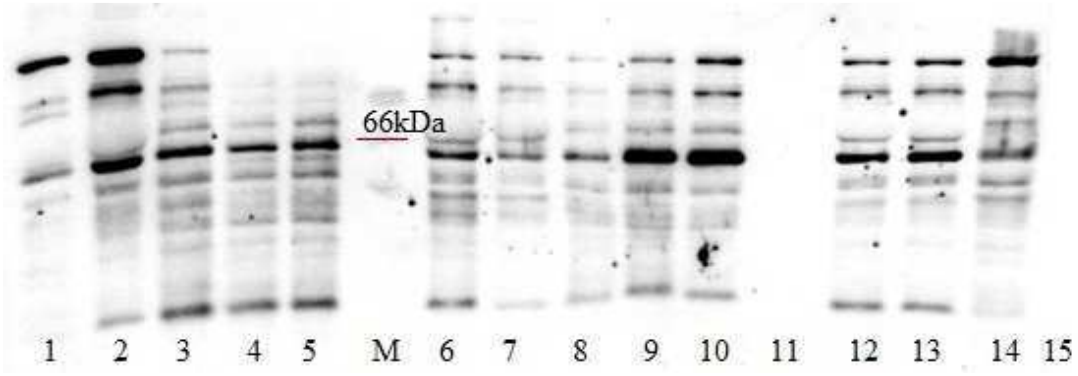


Figure 41, whole. Western blotting with anti-strep antibody of transformants t6 and t8. Lane 1: crude extract of t6; lane 2: supernatant of t6; lane 3: crude extract of t8; lane 4: supernatant of t8. Crude extracts samples were titrated to 20µg, while 10 µl of supernatants were added regardless of their protein concentration.



A

B

Figure 42, whole. A Western blotting with anti-strep antibody. **B** Coomassie staining. Lane1: "induced" SAA111 with 1%BSA crude extract; lane 2: induced t15 crude extract with 1%BSA; lane 3: induced t15 crude extract without BSA extracted with high salt buffer; lane 4:induced t15 crude extract without BSA extracted with low salt buffer and heparin; lanes 5: induced t15 crude extract without BSA extracted with low salt buffer; M: protein marker; lane 6: Flow through of sample in lane 2, purified with Strep-Tactin®; lane 7: washing 1 of sample in lane 2, purified with Strep-Tactin®; lane 8: Flow through of sample in lane 3, purified with Strep-Tactin®; lane 9 and 10: washings 1 and 2 of sample in lane 3, purified with Strep-Tactin®; lane 12: fraction 1 of sample in lane 3, purified with Strep-Tactin®; lane 13: sample of lane 2 treated with 1mM DTT for 96hours at -80°C; lane 14: sample of lane 2 treated with 1mM DTT for 96hours at -20°C; lane 15: pellet of another experiment. Samples were titrated to 20µg. Sample in lane 12 was not titrated.

APPENDIX 3

Pubblications:

Silvestrini L., Cervellini F., Severi I., Ortore M.G., Mazzola F., Galeazzi R., Amici A., Raffaelli N., Cianci M. (2019) Recombinant Expression of Human Lipoprotein Lipase in Lower Eukaryotes: Preliminary Results Filamentous fungi as microbial platform for "in solution" protein production. MICSA 2019 (Napoli). ISBN 978 88 8080 359 1.

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