ASPERGILLUS NIGER AS AN EXPRESSION SYSTEM FOR HETEROLOGOUS EXPRESSION OF ROL AND BTL2

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ASPERGILLUS NIGER AS AN EXPRESSION SYSTEM FOR HETEROLOGOUS PRODUCTION OF ROL AND BTL2

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ASPERGILLUS NIGER AS AN EXPRESSION SYSTEM FOR HETEROLOGOUS PRODUCTION OF ROL AND BTL2 LIPASES

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Key words: *Aspergillus niger*, lipase, BTL2, ROL, heterologous expression

Abstract

Lipases are esterases that hydrolyze lipids to fatty acids and glycerides. These enzymes have a variety of functions, thus applications, making them attractive targets to be studied. In this study, *Bacillus thermocatenulatus* lipase 2 (BTL2) and *Rhizopus oryzae* lipase (ROL) were cloned in pAL85 expression vector under control of constitutive pkiA promoter and trpC terminator. *Aspergillus niger* 872.11 protoplasts were transformed with these constructs. Transformation was confirmed by PCR from genomic DNA, lipase production was screened on Rhodamine-containing plates and positive strains were selected for shake flask cultures. Samples taken from the shake flask cultures were subjected to further analysis. SDS-PAGE and zymogram assay of BTL2 transformants showed that two transformants were expressing BTL2. Results of activity assay against 4-MU-caprylate and Bradford assay were also consistent with this data. But the results were not reproducible. Computational analysis showed that the enzyme was not suitable for high level expression in *A. niger*. No bands for ROL transformants could be detected on SDS-PAGE analysis. Activity assay against 4-MU-caprylate did not show a significant activity either. Differences in codon usage preferences between *Rhizopus oryzae* and *Aspergillus niger* were investigated in order to suggest an explanation for low efficiency.

ROL VE BTL2 LİPAZLARININ HETEROLOG ÜRETİMİ İÇİN BİR EKSPRESYON SİSTEMİ OLARAK *ASPERGILLUS NIGER*

Sedef Dinçer

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Özet

Bacillus thermocatenulatus lipazı (BTL2) ve *Rhizopus oryzae* lipazı (ROL), pAL85 plazmidinde, konstitütif promoter pkiA ve sonlandırıcı trpC dizilerin kontrolü altına klonlandı. Oluşturulan plazmidler, ipliksi bir mantar olan *Aspergillus niger* organizmasının 872.11 suşunu transforme etmek için kullanıldı. Transforme olan koloniler PCR ile doğrulandıktan sonra Rhodamine içeren katı besi yerinde pozitif sonuç veren suşların sıvı kültürleri yapıldı. Bu kültürlerden alınan örneklere çeşitli analiazler uygulandı. BTL2 ile transforme olmuş hücrelerde SDS jel ve zimogram sonuçları, BTL2'nun üretildiğini gösterdi. Aktivite ve Bradford testleri de bu sonuçlarla paralellik gösterdi fakat sonuçlar tekrar edilebilir değildi. Yapılan hesaplamalı analizler sonucunda bu proteinin *A. niger*'da yüksek seviyede üretim için uygunolmadığı görüldü. ROL ile transforme edilmiş suşlardan, SDS jelinde bant görülemedi. Aktivite testi de belirgin bir sonuç vermedi. Bu düşük verimin sebebini açıklamak üzere *Rhizopus oryzae* ve *Aspergillus niger*'in kodon kullanım tabloları incelendi.

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1. INTRODUCTION

1.1 Background Information on Lipases

Lipases were discovered in 1856 by Claude Bernard during his studies on pancreatic juice when an enzyme in the mixture caused oil droplets to dissolve. Initially, lipases were used in order to enhance digestion in humans and were obtained from animals. However, in time, shortage and inconvenience of these resources led to search of other organisms to be used instead of animals (Hasan, Shah, & Hameed, 2006). In 1901, Christiaan Eijkman showed that some bacterial species secreted lipases. This finding resulted in improvement of lipase studies although it was not until understanding of stability of lipases in organic solvents that many bacteria were tested in terms of lipase production. Eventually, lipases became a major class among the enzymes used in industry (Jaeger, Dijkstra, & Reetz, 1999). In 1981, first amino acid sequence determination study was done for pancreatic lipase (Decaro, et al., 1981). In 1990, first two 3D structures were determined for *Rhizomucor miehei* lipase and human pancreatic lipase (Verger, 1997).

1.2 Definition and function of lipases

Systematically, lipases are classified as triacyl glycerol hydrolases (E.C. 3.1.1.3). They act on carboxylic ester bonds in lipid molecules. Although they are called "hydrolases", under certain conditions lipases can catalyze either hydrolysis or synthesis of long chain triacyl glycerols (Gupta, Gupta, & Rathi, 2004). Commonly, lipases are identified as enzymes that are activated at oil-water interfaces (interfacial activation) since their substrates are insoluble in water and they have a lid-like structure on the molecule. Nevertheless, there are exceptional lipases that do not fit these criteria (Verger, 1997). Therefore, lipases are also defined as carboxyl esterases that hydrolyze

and synthesize long chain (containing ten or more Carbon atoms) acyl glycerols (Houde, Kademi, & Leblanc, 2004).

As mentioned above, lipases can catalyze synthesis reactions besides hydrolysis. These reactions can be grouped as follows: esterification, aminolysis, interesterification, alcoholysis (involves displacement of an acyl group between acyl glycerol and an alocohol), acidolysis (displacement of an acyl group between acyl glycerol and carboxylic acid); the last three of which are named together as transesterification reactions (Balcao, Paiva, & Malcata, 1996; Reis, Holmberg, Watzke, Leser, & Miller, 2009). Schematic view of lipase-catalyzed reactions is showed in Figure 1-1 (Houde, Kademi, & Leblanc, 2004). Parallel to variety of reactions, lipases have a wide range of substrates including aromatic, aliphatic, alicyclic and bicyclic esters (Chen & Sih, 1989).

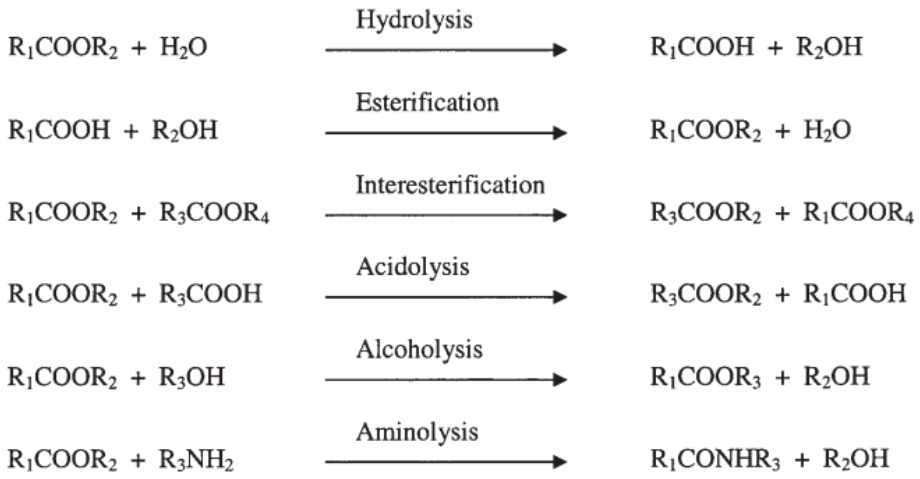

Figure 1-1**:** Reactions catalyzed by lipases

1.3 Structure of Lipases

The observations on lipase-catalyzed reactions showed that these enzymes were more active against aggregated substrates but until 1990, the underlying reason was not known (Brady, et al., 1990; Winkler, Darcy, & Hunziker, 1990). Since human pancreatic lipase and *Rhizomucor miehei* lipases had their 3D structures determined, first conclusions and predictions were made according to these structures. It was seen that a domain was covering the active site, blocking its interaction with the solvent. This finding led to a theory that this domain could be functioning as a lid, going through a conformational change in certain conditions, and providing substrate access to the

active site. This theory was confirmed with additional co-crystallization studies. In the following years, other X-Ray studies showed that lipases belong to α/β hydrolase fold family. This type of fold characteristically contains a hydrophobic β-sheet consisting of eight stands in the core, surrounded by up to six layers of amphiphilic α-helices (Lang & Dijkstra, 1998). The active site consists of three residues (catalytic triad): serine, aspartic acid or glutamic acid and histidine. The nucleophilic serine residue of the catalytic triad is located on the C- terminus of the fifth β-strand in a conserved sequence as GXSXG and this pentapeptide forms the so-called nucleophilic elbow, structurally a β-turn-α motif (Hide, Chan, & Li, 1992; Jaeger & Reetz, 1998). From the other two residues of the active site, glutamic or aspartic acid is located after the seventh β-strand and the glycine is located between eighth β-strand and the sixth α-helix. Topology of the α/β fold with the active site residues are shown in Figure 1-2 (Jaeger, et al., 1999).

Position of the active site with respect to the lid on the enzyme differs among species and this property is categorized into three groups. The first class of enzymes has both the lid and the active site on the surface of the protein which is the case for *Thermomyces lanuginosa* lipase. Another class of lipases has a funnel shaped lid towards the active site which corresponds to *Candida antarctica* lipase. Finally, lipases belonging to the third group have a tunnel-like lid and their active site at the end of this tunnel. *Candida rugosa* lipase is an example for the last class (Gutierrez-Ayesta, Carelli, & Ferreira, 2007).

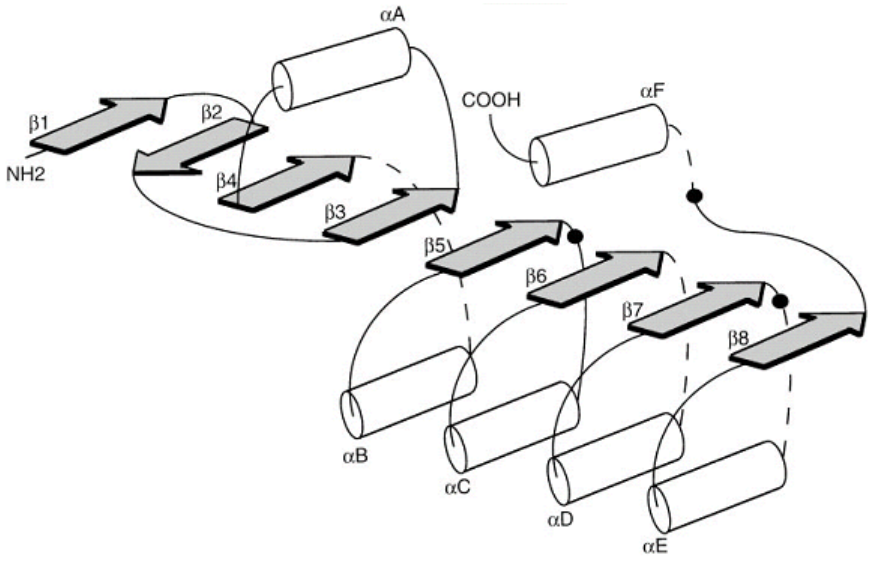

Figure 1-2: α/β hydrolase fold

1.4 Mechanism of action of lipases

Activation of lipases occurs at a critical micellar substrate concentration, thus called interfacial activation. Upon contact to this interface, the lid or lids of the lipase change in fold in a way that the active site, which is an elongated hydrophobic cavity that acyl groups can bind, becomes accessible (Svendsen, 2000).

After the substrate has bound to the active site, reaction mechanism takes place at five steps (Figure 1-3 (Jaeger, et al., 1999)). First, a nucleophilic attack occurs from the oxygen atom of the hydroxyl group of the nucleophilic serine residue to the carbonyl group on the ester bond of the lipid. This attack makes the carbonyl oxygen an oxyanion, being transiently stabilized by one or more hydrogen bonds, forming an oxyanion hole. This nucleophilic attack is partly enhanced by the catalytic histidine residue by transferring a proton from the serine residue. Subsequntly, the proton is transferred to the ester oxygen and the ester bond is cleaved. The alcohol moiety leaves while the acid component of the substrate is bound to the serine residue, forming the covalent intermediate. In the next step, active site histidine draws a proton from a water molecule and the resulting OH- group attacks the carbonyl carbon atom of the acyl group bound to serine. Again an oxyanion hole forms to stabilize the transient state. Finally, the histidine residue donates a proton to the oxygen of serine residue, causing it to release the acyl group (Cygler, et al., 1994; Jaeger, et al., 1999).

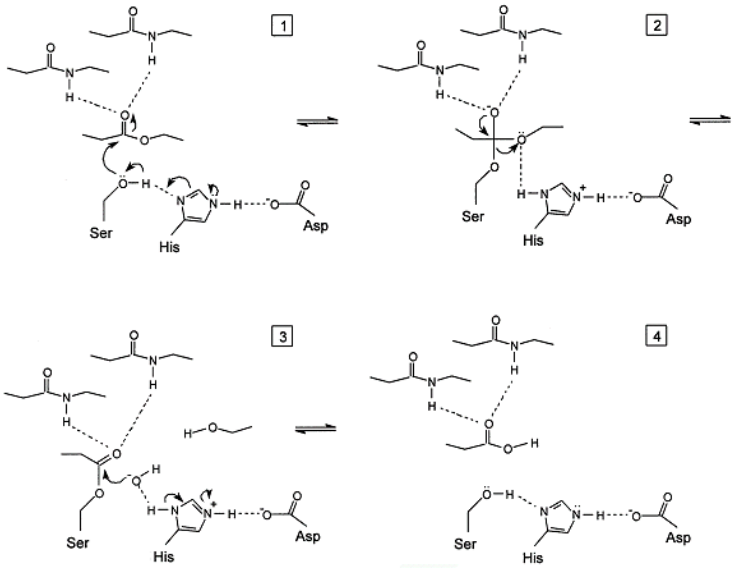

Figure 1-3: Reaction steps

Since lipases are activated at lipid-water interfaces, the reactions described above cannot be explained by Michaelis-Menten equations as they are applicable to homogenous state reactions only (Jaeger & Reetz, 1998).

1.5 Substrate selectivity of lipases

Naturally, different lipases possess different levels and types of selectivity. Although they have a wide substrate and reaction range, rate of reaction varies according to structure of substrate, factors affecting binding and other conditions like temperature and type of solvent (R. G. Jensen, Dejong, & Clark, 1983; Reetz, 2002; Reis, et al., 2009). Nevertheless, almost every lipase exhibits a degree of selectivity against carboxylic acid while the most extraordinary example is *Geotrichum candidum* lipase as it selectively attacks fatty acids with cis-9 configuration (R. G. Jensen, et al., 1983; R. G. Jensen, Galluzo, D. R., Bush, V. J., 1990). Steric hindrance and hydrophobic interactions are the most widely accepted properties determining selectivity of a lipase against alcohols and carboxylic acids (Bevinakatti & Banerji, 1988; Brockerhoff, 1968; Cygler, et al., 1994).

Applications of a lipase are often related to its substrate selectivity. Most of the microorganisms express more than one lipases that differs in terms of selectivity. For instance, *Mucor miehei* lipase at acidic pH specifically hydrolyzes milk fat in order to produce butyric acid whereas hydrolysis of tributyrin occurs slowly by some other microbial lipases (Bjorkling, Dahl, Patkar, & Zundel, 1994; Moskowitz, Cassaigne, West, Shen, & Feldman, 1977; Sugiura & Isobe, 1975). For substrates carrying an alcohol group, lipases show both regio- and stereospecificity (Chapman, 1969). Based on their regiospecificities, lipases can be classified as follows (Macrae & Hammond, 1985).

First group of enzymes are defined as lipases that hydrolyze mono- and diglycerides as well as triglycerides, therefore not causing accumulation of intermediate products. Candida cynlindracea lipase belongs to this group (Benzonana & Esposito, 1971).

Second group lipases, on the other hand, carry out hydrolysis from the outer 1- and 3- positions. As a result, they produce fatty acids, 1,2-diacylglycerol and 2-monoacylglycerol (Macrae & Hammond, 1985). This type of regiospecifity is found in *Rhizopus arrhizus*, *Rhizopus japonicus*, *Humicola lanunignosa* and some others (Semeriva, Benzonana, & Desnuelle, 1967).

In addition to these, lipases from *R. arrhizus, R. delemar, C. cylindracea,* and *P. aeruginosa* were shown to be partially stereospecific; therefore found to be useful in isolation of optically pure esters and alcohols (Cambou & Klibanov, 1984).

1.5.1 Factors affecting selectivity of lipases

Stability of lipases in organic solvents is a great advantage for reactions of synthetic organic chemistry. However, reduced activity of the enzyme and reversibility of reactions are problems that are trying to be overcome (Klibanov, 2001). One strategy applied was to add vinyl acetate as acylating agent to reaction medium. This resulted in formation of acetaldehyde, preventing the irreversible reaction. Disadvantage of this approach is that acetaldehyde deactivates some lipases, which limits application (Villeneuve, Muderhwa, Graille, & Haas, 2000).

Temperature is one of the most determining conditions of an enzymatic reaction. Thus, it alters selectivity of lipases. A common knowledge is that lowering the temperature, despite slowing down the reaction, increases enantioselectivity. This theory is supported by a kinetic resolution reaction catalyzed by *Thermomyces lanuginosus* lipase. As temperature was decreased from 40 ^{o}C to -20 ^{o}C, enantioselectivity factor, E, increased from 13 to 84 (Lopez-Serrano, Wegman, van Rantwijk, & Sheldon, 2001). However, this is not the case for all reactions. In a lipase-catalyzed esterification reaction, enantioselectivity factor increased from 13 to 120, when temperature was elevated to 57 ^{o}C from 37 ^{o}C (Watanabe et al., 2001).

The solvent is also of great importance for an enzyme to work efficiently. For example, pretreatment of lipases with an organic solvent before the actual reaction, prevents the time lag (Matsumoto, Kida, & Kondo, 2001). Use of ionic liquids as reaction solvent is still another modification to enhance enantioselectivity of kinetic

resolution, enzyme stability, and sustainable activity (Schofer, Kaftzik, Wasserscheid, & Kragl, 2001).

1.6 Applications of lipases

Lipases are one of the largest groups of enzymes exploited industrially due to their following properties: They are able to catalyze not only hydrolysis but also synthesis of triglycerides (Jaeger, et al., 1997); they have a wide substrate range and they can catalyze a number of different reactions (Houde, et al., 2004); they exhibit high substrate specificity, enantoiselectivity, stereoselectivity and regioselectivity (Naik, et al., 2010) they operate under mild reaction conditions; they do not require cofactors for hydrolytic reactions; they are stable in organic solvents (Hasan, et al., 2006).

The fields that involve lipases as catalysts are mainly detergent industry, fats and oils, food and dairy industry, paper industry, leather industry, textile industry, biodiesel industry, surface cleaning, oleochemical industry, synthesis of fine chemicals, medical applications, cosmetics and organic synthesis reactions (Reetz, 2002).

1.6.1 Detergent industry

Detergent industry is the biggest market for application of lipases (Saxena, et al., 1999). The three requirements an appropriate lipase to be used as detergent additive must need are: low substrate specificity in order to hydrolyze a wide range of fats and oils, stability in harsh washing conditions such as basic pH values and high temperatures, ability to resist damaging detergent additives like surfactants (Jaeger & Reetz, 1998). First commercial lipase produced for detergent industry was called Lipolase, by Novo Nordisk in 1988 (Houde, et al., 2004).

1.6.2 Food industry

In food industry, lipases are used to modify fatty acids in terms of location, chain length and degree of unsaturation. These parameters not only affect physical properties

of the food but they also change nutritional values and taste of a given product (Jaeger & Reetz, 1998).

Case of cocoa butter is an example of lipase application in food industry. Cocoa butter includes palmitic and stearic acids, has a melting point of 37 ^{o}C and used in products like chocolate since it gives a desirable cooling sensation when melts in mouth. A lipase-based technology is used for transforming less valuable fats to cocoa butter constituents (Undurraga, Markovits, & Erazo, 2001).

Polyunsaturated fatty acids (PUFAs) which are taken by diet, are essential for humans in membrane and prostaglandin synthesis. They are also used in pharmaceutical industry for production of antiinflammatories. Lipases are used to obtain PUFAs from plant animal lipids (Gill & Valivety, 1997).

Other applications of lipases in food industry include the lipolysis reaction which involves removal of fat from meat, fermentative steps of sausage production and earlier, rice, soybean milk and wine processing involved lipase-catalyzed steps as well (Houde, et al., 2004).

1.6.3 Dairy industry

Infant formulas are among the products that are being processed and improved by lipases. They serve as important alternatives to breast milk, although palmitic acid in breast milk is more easily absorbed due to its location on the triglyceride. Majority of the triglycerides in breast milk are saturated at *sn*-2 position and unsaturated at *sn*-1,3 positions. During digestion, pancreatic lipases hydrolyze *sn*-1,3 fatty acids selectively, leaving the palmitic acid at *sn*-2 position. Since palmitic acid groups in infant formulas are not specifically on *sn*-2 position, digestion of these products liberates palmitic acid, which binds to calcium, leading to poor absorption and constipation. Therefore, lipases are used on infant formulas in order to obtain saturated fatty acids at *sn*-2 position (Sellappan & Akoh, 2002).

Cheese ripening is another process in which lipases are exploited. Features like texture and aroma depend on the fat content and products of fat degradation. Exogenous lipases added to cheese usually accelerates ripening process but addition of free enzyme causes undesirable effects. A strategy developed to prevent these defects is

encapsulation of the lipase (Kheadr, Vuillemard, & El-Deeb, 2003; Kheadr, Vuillemard, & El-Deeb, 2002). Addition of lipase to the cheese results in release of short chain fatty acids, generating the sharp flavor of cheese and medium chain fatty acids generate a soap-like taste in cheese (Saxena, et al., 1999).

As the name indicates, enzyme-modified cheese (EMC) is also produced using enzymes, including lipases. In this industrial process, cow milk is treated with lipases in order to obtain a flavor that imitates ewe or goat milk. EMCs are used in food like sauces as well (Houde, et al., 2004).

1.6.4 Textile industry

Desizing materials used on different fabrics like cotton and denim, include lipases as well as estereases. In addition to desizing, lipases also take place in lubrication, abrasion and dye absorption systems (Hasan, et al., 2006).

1.6.5 Paper industry

Hydrophobic content of wood (pitch) that is mainly composed of triglycerides and waxes, inhibit paper manufacturing process (Jaeger & Reetz, 1998). Lipases are used in paper industry to hydrolyze those compounds. Since the beginning of 1990s, enzymatic pitch-control has been a routine process in paper industry (Bajpai, 1999). Commercially, *Candida rugosa* lipase is being used in Japan, hydrolyzing up to 90 % of wood triglycerides (Sharma, Chisti, & Banerjee, 2001). Besides hydrolyzing hydrophobic components, lipases are also exploited in deinking of waste papers which in turn leads to saving energy, time, costs etc. Lipase from Pseudomonas species is an example of enzymes used for this purpose.

1.6.6 Organic synthesis

Thanks to their following properties, lipases have gained great importance in organic chemistry: Since they naturally bind acyl moieties, lipases have large domains

and a wide susbstrate range of synthetic chemicals without losing their regio- and enantioselectivity. Secondly, they have unusually stable structures that withstand oil/water interfaces which provide them the ability to remain intact in organic solvents. Thirdly, lipid hydrolysis is easily reversed in nonaqueous media, being interesterification or ester synthesis. Lastly, lipases serve as selective acylating agents towards nucleophiles (Louwrier, Drtina, & Klibanov, 1996). The most important disadvantage of lipase selectivity is against acyl group of esters, most lipases accept aliphatic groups rather than bulky, aromatic groups.

In organic media, lipases are most widely used for their enantioselectivity, the main usage being resolution of racemic compounds (Martin et al., 1981). The three groups of lipase-catalyzed reactions in organic solvents are: resolution of racemic alcohols, resolution of racemic acids, regioselective acylations.

Candida cylindracea lipase is used in a biphasic system for resolution of racemic alcohols. Transesterification reactions were carried out in diethyl ether and heptane with porcine pancreatic lipase (Kirchner, Scollar, & Klibanov, 1985).

Candida cylindracea lipase was also applied in resolution of racemic acids. It was used to separate enantiomers of 2-bromopropionic acids and 2-chloropropionic acids. These acids are starting compounds in synthesis of some herbicides (Kirchner, et al., 1985). *Pseudomonas fluorescence* lipase is another enantioselective lipase used in treatment of racemic acids. It was used in order to carry out asymmetric ring opening reactions (Yamamoto, Nishioka, Oda, & Yamamoto, 1988). Again, porcine pancreatic lipase was used in this type of reactions.

Lipases express regioselectivity in acylation of steroids, sugar derivatives and sugars (Therisod & Klimanov, 1987). Lipases are also able to acylate hydroxyl groups on glycals, regioselectively. Acylation steps in synthesis of hydroxy steroids are other examples of lipase-catalyzed organic synthesis (Riva, Bovara, Ottolina, Secundo, & Carrea, 1989).

1.6.7 Synthesis of fine chemicals

1.6.7.1 Pharmaceutical industry

Drug molecules need to be enantiopure because only one enantiomer of a certain chemical gives the desired effect with minimal undesirable side effects. As stated earlier, lipases exhibit regioselectivity and enantioselectivity. They are able to function in mild conditions that prevent racemization, isomerization. They can be reused as immobilized biocatalysts and they provide an economic process. Due to these advantageous features of lipases, they have great importance in pharmaceutical industry (Houde, et al., 2004).

Some lipases are useful for synthesizing enantiopure alcohols, amides, carboxylic acids and esters which are found in antiiflammatory, antiviral, anticancer, anti-Alzheimir disease, anti-cholesterol drugs and vitamin A (Bonrath, Karge, & Netscher, 2002; Houde, et al., 2004). Paclitaxel, an anticancer drug that inhibits mitosis by preventing microtubule depolymerization, is used for treatment of ovarian cancer and metastatic breast cancer. The chemical was initially being extracted from yew tree *Taxus brevifolia* but the yield was low. Alternative method for obtaining paclitaxel is coupling baccatin III (paclitaxel without C-13 side chain) or 10-deacetylbaccatin II (paclitaxel without C-13 side chain and C-10 acetate) with C-13 paclitaxel side chain. Baccatin III and 10-deacetylbaccatin II required for this semi synthetic reaction can be obtained from young Taxus cultivars or shoots, without cutting trees. C-13 paclitaxel side chains were obtained from the enantioselective hydrolysis of racemic acetate-cis-3-(acetoxy)-4-phenyl-2-azetidione to only corresponding (3S)-alcohol and desired (3R)-acetate. Lipase PS-30 from Pseudomonas cepacaia or Bristol-Myers Squibb lipase from Pseudomonas species was used for catalysis of this hydrolysis (Patel, 1998).

A nonsteroidal anti-inflammatory named Ibuprofen (2-(4-isobutylphenyl) propionic acid) was also synthesized by lipase-catalyzed reactions. This compound inhibits binding of arachidonic acid and prevents prostaglandin synthesis that act on anti-inflammatory response. Ibuprofen consists of two enantiomers, the (S)-ibuprofen being 160 times more effective against prostaglandin synthesis compared to (R)-ibuprofen. To separate these enantiomers, specific lipases were used in order to carry out the esterification reaction of (S)-enantiomer with methanol or butanol, forming the

(S)-ester which is subsequently removed from (R)-ibuprofen and transformed to the desired final product (S)-ibuprofen (Houde, et al., 2004; Sharma, et al., 2001).

Another therapeutic compound that represents application of lipases is diltiazem, a calcium channel blocker. In synthesis of diltiazem, lipases take place in resolution of racemic epoxyesters. The enantioselective hydrolysis is done by *Serratia marcescens* lipase. The desired product of this reaction is then converted to diltiazem (Houde, et al., 2004).

1.6.7.2 Cosmetics industry

Burkholderia cepacia lipase was used for the transesterification step in order to obtain enantiomerically pure (-)-menthol. The final product menthyl methacrylate was then used in a perfume (Athawale, Manjrekar, & Athawale, 2001). Another perfume component, (-)-methyl jasmonate, originally a plant growth factor, is also synthesized by a lipase-catalyzed reaction, using a commercially available lipase preparation Lipase P (Kiyota, Higashi, Koike, & Oritani, 2001).

Immobilized *Rhizomucor miehei* lipase was used as a catalyst for synthesis of cosmetic additive fatty acids like isopropylmyristate, isopropylpalmitate and 2-methylhexylpalmitate. These compounds were then used in cosmetic personal care products like sun creams, bath oils etc. This strategy resulted in products of higher quality and reduced the following refining steps (Hasan, et al., 2006).

Vitamin A and derivatives (Retinoids) also have great importance in cosmetics industry especially in skin and hair care products. Immobilized lipases were used for these products in order to water-solubilize retinol (Maugard, Rejasse, & Legoy, 2002).

1.6.7.3 Agrochemicals

To prevent growth of grass weeds on paddy fields, a novel herbicide named (S)-idanofan was produced in an enantiopure manner using lipase-catalyzed reactions (Tanaka, Yoshida, Sasaki, & Osano, 2002). In another study on agrochemical, lipase B

from *Candida antarctica* was used for enantioselective hydrolysis of racemic 4-oxo-1,2-pyrrolidinedicarboxylic acid dimethyl ester, producing cis-4-hydroxy-D-proline or trans-4-hydroxy-D-proline (Sigmund, Hong, Shapiro, & DiCosimo, 2001).

For production of phenoxypropionate herbicides, porcine pancreatic lipase in anhydrous hexane was used in as the catalyst to carry out the selective esterification of (S)-isomers with butanol (Saxena, et al., 1999).

1.6.7.3 Biodiesel

Biodiesel production is a field that uses plant-originated oils and produces short chain alcohol esters. Lipases in organic solvents are able to catalyze this reaction in one step called transesterification. Although production in industrial scale has not been achieved, two strategies have been developed, one making use of *Rhizopus oryzae* lipase in a solvent-free reaction system (Parawira, 2009).

1.7 *Bacillus thermocatenulatus* lipase 2

One of the two lipases produced by the thermophile organism *Bacillus thermocatenulatus*, BTL2, is considered to be promising for industrial applications (Schlieben, Niefind, & Schomburg, 2004). It was first characterized by using *Escherichia coli* expression system in 1996 (SchmidtDannert, Rua, Atomi, & Schmid, 1996). This first study showed that the enzyme has a molecular weight of 43 kDa, optimum working temperature and pH range are 60-70 ^{o}C and pH 8-9, respectively. Moreover, it showed significant stability between pH 9-11 as well as in a number of detergents and organic solvents. As mentioned above, these properties are advantageous for applications in industrial and biotechnological fields. BTL2 has been functionally expressed in *Eschericia coli* and *Pichia pastoris* (Quyen, Schmidt-Dannert, & Schmid, 2003; Rua, Atomi, Schmidt-Dannert, & Schmid, 1998; Rua, SchmidtDannert, Wahl, Sprauer, & Schmid, 1997).

1.8 *Rhizopus oryzae* lipase

Lipases of genus Rhizopus are valuable for chemical synthesis due to their positional selectivity against sn1 and sn3 locations and primary alcohol esters (Henke, Schuster, Yang, & Bornscheuer, 2000). This property of these enzymes make theme preferred enantioselective catalysts for pure production of fine chemicals (Demir, Hamamci, Tanyeli, Akhmedov, & Doganel, 1998). Lipase from *Rhizopus oryzae* (ROL) is one of these enzymes for which optimum conditions are 40 ^{o}C temperature and pH 8.5 (Henke, et al., 2000). Initial studies on expression of ROL were in *E. coli*. Although the expressed ROL enzyme was inactive as inclusion bodies, they were refolded in additional experiments (Di Lorenzo, Hidalgo, Haas, & Bornscheuer, 2005). Other than *E. coli,* ROL was heterologously expressed in *Pichia pastoris* and *Saccharomyces cerevisea* (Di Lorenzo, et al., 2005; Minning, Schmidt-Dannert, & Schmid, 1998; Resina, Serrano, Valero, & Ferrer, 2004; Takahashi, et al., 1998).

1.9 *Aspergillus niger*

In industry, a common approach for large scale production is to use filamentous fungi since these organisms are capable of producing and secreting extremely high amounts of homologous proteins. Apergillus species are widely used in this field. Under optimized fermentation conditions, *Aspergillus niger* secretes 20 g/l glucoamylase (Iwashita, 2002). *A. niger* is the most preferred fungus amongst Aspergilli. As some other Aspergillus species, it is Generally Regarded As Safe (GRAS) and exploited in a number of industrial applications, with a considerable fraction being on food industry (Schuster, Dunn-Coleman, Frisvad, & van Dijck, 2002). The major usage of *A. niger* in industry is citric acid production, with a yield of more than one million metric tons per year (Baker, 2006). Certain properties of filamentous fungi, thus of *A. niger* too, like performing post-translational modifications correctly, growing on a relatively cheap medium and stabilizing the heterologous gene by insertion into the genome make them promising hosts for protein expression (Wang, Ridgway, Gu, & Moo-Young, 2005). However, heterologous and non-fungal genes are expressed with a lower yield compared to homologous and fungal genes in filamentous fungi. Therefore, various strategies have been developed to improve this system. One of these improving methods

is gene fusion. It is based on fusing the gene of interest to the 3' end of the signal sequence of a highly produced and secreted gene, usually glucoamylase in *A. niger*. By this method, from 5 to 1000 fold increase, reaching 250 mg/l in heterologous expression of different genes have been achieved in Aspergillus species hosts (Gouka, Punt, & vandenHondel, 1997). To avoid degradation of secreted proteins, protease-deficient Aspergillus strains and protease inhibitors are used because extracellular proteases are at high level in wild type Aspergilli (van den Hombergh, van de Vondervoort, Fraissinet-Tachet, & Visser, 1997). In addition to these strategies, regular conditions like pH, temperature, aeration etc. are also being optimized to increase efficiency of expression (Lubertozzi & Keasling, 2009).

1.9.1 Morphology

Aspergillus niger cells form white mycelium on solid media that usually begin sporulating after two days. Spores are dark brown, globular, 3.5-5 μm in diameter and carried on conidiophores. Conidiophores are smooth-walled, hyaline, turning dark towards the spore-carrying vesicle (Figure 1.4) (Abarca, Accensi, Cano, & Cabanes, 2004).

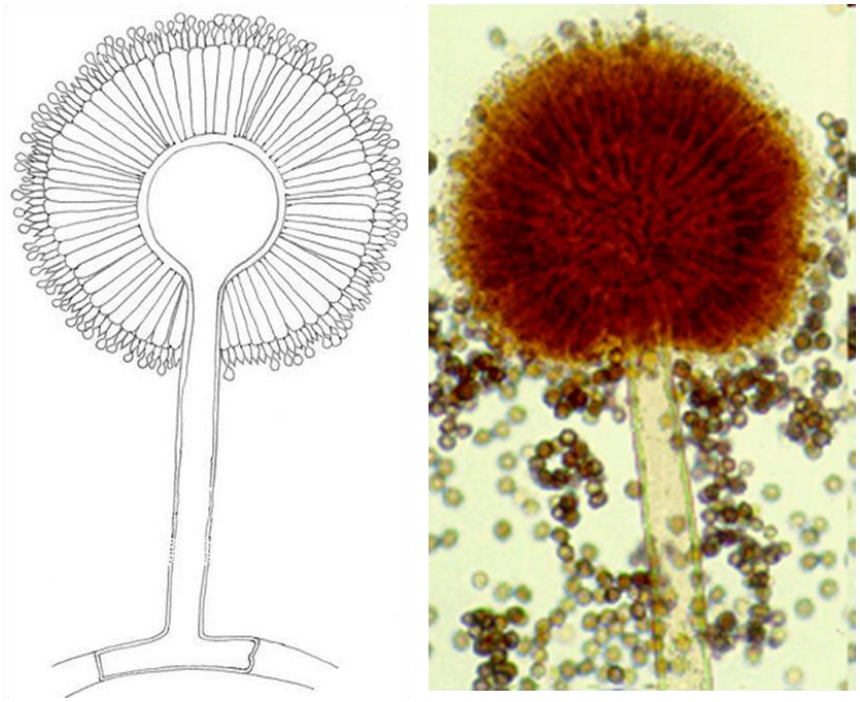

Figure 1-4: Conidiophore and spores of *A. niger*

1.9.2 Secretory pathway

To explain targeting of protein to endoplasmic reticulum (ER), in *Saccharomyces cerevisiae*, two pathways were elucidated. One of these is by means of the signal recognition particle (SRP) while the other one is SRP-independent. Since homologue of *S. cerevisiae* SRP was identified in *A. niger*, it is accepted that these routes are present in filamentous fungi too (Conesa, Punt, van Luijk, & van den Hondel, 2001; Pritchard, et al., 1995). Either by SRP or cytoplasmic chaperons, the protein folding is interrupted until arrival to ER. Therefore, maturation of the polypeptide continues in ER lumen again by the help of chaperons and foldases. These helper proteins include Binding Protein (BiP), Protein Disulfide Isomerase (PDI), Peptidyl Prolyl Isomerase (PPIase) and Calnexin. BiP functions in transporting the polypeptide to ER, protein folding and assembly mechanism and degradation of misfolded proteins (Pedrazzini & Vitale, 1996) 1996). PDI, as the name indicates, has role in arrangement of disulfide bonds on the newly synthesized protein. It catalyzes reduction, oxidation and isomerization of disulfide bonds (Noiva, 1999). Although PPI has proven to accelerate protein folding in vitro, its function is not clear. However, it is known that it catalyzes isomerization of cis trans peptide bonds on N-terminal of proline residues (Gothel & Marahiel, 1999). Calnexin is especially important for maturation process of glycoproteins. Calnexin specifically binds monoglycosylated proteins and with assistance of glucosidase II, protein is folded and correctly glucosylated (Jakob & Burda, 1999).

Control of folding by ER, involves two mechanisms: Unfolded protein response (UPR) and ER-associated degradation of proteins (ERAD). UPR mechanism is based on presence of unfolded proteins in the ER, resulting in increased synthesis of chaperons. ERAD, on the other hand, degrades proteins in the cytoplasm that cannot be folded properly (Conesa, et al., 2001).

Once the folding is succeeded, proteins are targeted to Golgi apparatus. In filamentous fungi, however, a Golgi-like structure exists instead of the Golgi apparatus, functioning the same way. Peptidase reactions and glycosylation modifications take place at this step of the pathway (Archer & Peberdy, 1997; Jalving, van de Vondervoort,

Visser, & Schaap, 2000). Finally, the proteins are either secreted from the cell membrane or targeted to vacuoles (Bryant & Stevens, 1998).

1.10 Methodological Background

1.10.1 4-Methylumbelliferone assay

Fluorogenic assays involve detection of increase in fluorescence as the substrate is being hydrolyzed. The advantages of these methods are that they are highly sensitive (depending on the specific activity of the lipase of interest on a particular substrate), they are affected less by compounds that cause background signals (Schmidt & Bornscheuer, 2005), and the reaction products can be monitored continuously (Gilham & Lehner, 2005).

Using esters of 4-methylumbelliferone is a type of fluorescence assays to measure lipase activity. Once the ester linkage is cleaved, the product becomes highly fluorescent (Jacks & Kircher, 1967). There is a wide range of 4-methylumbelliferyl esters available (Gilham & Lehner, 2005).

1.10.2 Rhodamine assay

Fluorescent dye Rhodamine B is used to visualize lipase activity although the underlying mechanism is not clear. Kouker and Jaeger were first to formulate a Rhodamin B-containing solid medium to screen lipase production by colonies (Kouker & Jaeger, 1987). This medium contained olive oil as substrate. Both growing cells and culture supernatant of lipase-producing organisms gave positive results when observed at 350 nm wavelength. Authors also stated that this method was able to detect 1 nkat of lipase activity whereas titrimetric assay required at least 20 nkat.

1.10.2 Strain & Plasmid

1.10.2.1 Choice of signal sequence and promoter

For secretion of the heterologous gene product, use of an efficient signal sequence is a common strategy (Punt, et al., 2002). In case of *Aspergillus niger*, secretion signal of glucoamylase gene is a promising alternative for this purpose since it is produced and secreted in high amounts by *Aspergillus niger*, naturally (Cullen, et al., 1987).

pAL-85 plasmid contains pkiA promoter which is a constitutive promoter that is active regardless of the carbon source in the media (Roth & Dersch, 2010).

pyrA gene product, carbamylophosphate synthetase (Lerner, Stephenson, & Switzer, 1987) is involved in arginine and pyrimidine synthesis pathway. Schematic view of the pathway is shown in Figure 1.5 (Bussey & Ingraham, 1982). The strain used in this study is mutant in pyrA gene, therefore pyrA on plasmid is used as a selection marker when cells can grow on media without uridine.

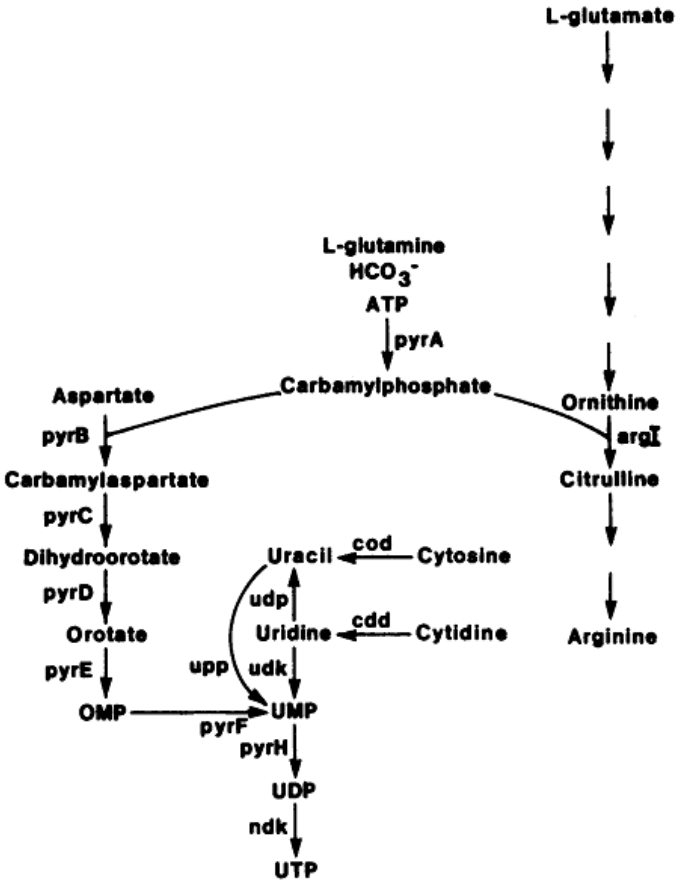

Figure 1-5: Arginine and pyrimidine biosynthetic pathway

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals used for this study are listed in Appendix.

2.1.1.2 Buffers and solutions

Standard molecular biology laboratory buffers and solutions were prepared according to protocols in Molecular Cloning: A Laboratory Manual, Sambrook et al. 2001.

2.1.2 Media

LB medium used for bacteria was prepared according to the protocol in Molecular Cloning: A Laboratory Manual, Sambrook et al. 2001.

For *Aspergillus niger*; Minimal Medium, Transformation Medium, MMS, MMS-Top media were used. Compositions of these media are listed in Appendix.

2.1.3 Molecular biology kits

Commercial kits and suppliers are listed in Appendix.

2.1.4 Primers

Primers were purchased from Microsynth (CH). Sequences are given in the appendix.

2.1.5 Plasmids

pAL-85 was obtained as a kind gift from Leo de Graaff (Wageningen University, Netherlands). Vector map of pAL-85 is given in Appendix.

2.1.6 Strains

Aspergillus niger 872.11 strain was used for expression. *E. coli* XL-1 Blue strain was used for cloning studies.

2.1.7 Enzymes

Commercial enzymes used for restriction digestion and other purposes are listed in Appendix.

2.1.8 Equipment

Laboratory equipment used in this study are listed in Appendix.

2.2 Methods

2.2.1 Cloning of BTL2 and ROL genes into pAL85 vector

2.2.1.1 PCR

BTL2/ROL gene was amplified by PCR using primers F_BTL2_S/F_ROL_S and R_BTL2_S from pPICZalphaA-BTL2/ROL plasmid which was previously constructed. PCR conditions with Taq polymerase were as follows: Initial denaturation at 94 ^{o}C for 10 minutes, followed by 34 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 54 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1 minute. Final extension was done at 72 ^{o}C for 7 minutes. PCR reaction was run on 1 % agarose gel and PCR product was extracted from gel using Qiagen Gel Extraction kit.

Next, another PCR was done with this PCR product using Sense_SS and R_BTL2_S primers. Reaction conditions were same as the one before except for the annealing temperature (53 ^{o}C). This reaction, too, was run on 1 % agarose gel and PCR product was extracted from gel.

Finally, product of the last PCR was used as template in a third PCR reaction with primers F_SS and R_BTL2_S/ROL_S under same conditions. PCR product was again isolated from agarose gel.

2.2.1.2 Digestion

pAL-85 was digested with NdeI and NotI according to manufacturer's instructions. Then the reaction mixture was treated with SAP again according to instructions supplied by Fermentas. Finally the digestion product was extracted from agarose gel.

2.2.1.3 Ligation

In-fusion PCR cloning reaction for BTL2 insert and pAL-85 vector was performed according to manufacturer's instructions.

2.2.1.4 Transformation

Preparation of chemically competent *Escherichia coli* XL-1 Blue cells to be used for transformation was carried out according to the protocol in Molecular Cloning: A Laboratory Manual, Sambrook et al., 2001.

For transformation, competent cells were first thawed on ice. When thawing was complete, 200 µl of cells were added on the ligation mixture and tubes were kept on ice for 30 minutes. Following this incubation, heat shock was performed by a 42 ^{o}C heater for 90 seconds. Then tubes were cooled on ice again for a few minutes and 750 µl of SOC medium was added in the tubes. Next, cells were incubated in a 37 ^{o}C shaker for 1 hour. Finally, cells were pelleted bu centrifugation at 6000 rpm for 3 minutes, 8500 µl of supernatant was discarded, remaining 100 µl was spread on LB-Kanamycin plates and left for overnight incubation at 37 ^{o}C.

2.2.1.5 Colony PCR

All colonies from both transformation plates were selected and colony PCR was done using Taq polymerase and F_seq_ pAL85 and R_seq_pAL85 primers.

For this reaction, first, a very small amount of cells from the colony was taken with a small micropipette tip. And the cells taken were spread on the walls of a PCR tube. Then 5 µl of ddH_2O was added to each tube and tubes were incubated at 94 ^{o}C for 5 minutes. Then, PCR Master mix was added and the reaction was started. Reaction conditions were the same as the one described above.

2.2.1.6 Culture Growth

Colonies that gave positive PCR result were picked from the plates and inoculated in 6 ml LB medium containing 50 μg/ml Kanamycin and grown overnight at 37 ^{o}C shaker.

2.2.1.7 Plasmid Isolation

Isolation of plasmid from liquid cultures was performed using Qiagen Miniprep kit, according to manufacturer's instructions.

2.2.1.8 Restriction and Sequencing Analyses

Isolated plasmids were digested with NdeI and NotI, according to the protocol by Fermentas and run on 1 % agarose gel. Finally, constructs were confirmed by sequencing.

2.2.2 Transformation of *Aspergillus niger*

2.2.2.1 Preparation of protoplasts

250 ml of Transformation medium was inoculated with *Aspergillus niger* 872.11 spores at a final concentration of 10^6 spores/ml and grown overnight at 30 ^{o}C shaker, 250 rpm. After incubation, mycelia were harvested by suction on a Büchner funnel and nylon gauze and washed with SMC solution. Then 1 g cells were re-suspended in 10 ml SMC, containing 0.1 g Lyzing enzymes from *Trichoderma harzianum* and suspension was incubated at 30 ^{o}C with by shaking at 100 rpm. After 1 hour, a sample was taken to

count and check protoplast formation. When protoplast concentration reached approximately 6.10^8 protoplasts/ml were present, mixture was filtered over nylon gauze, filtrate was collected and centrifuged at 2000 rpm and 4 ^oC for 10 minutes. Pellet was re-suspended in 5 ml STC solution and centrifuged for one more time and again re-suspended in STC. Finally, concentration was checked under microscope, STC was added to make the concentration 10^8 protoplasts/ml, aliquots were made and stored at -80 ^oC.

2.2.2.2 Transformation

Protoplasts were taken from -80 ^oC refrigerator and thawed on ice. 1 µg pAL85-BTL2 dissolved in 20 µl TE was added on 200 µl of protoplasts. Then 50 µl PEG was added and the mixture was incubated at room temperature for 20 minutes. When incubation was complete, 2 ml PEG solution was added and again incubated at room temperature for 5 minutes. Next, 4 ml STC was added and finally 30 ml selective MMS-Top agar was added in the tube and poured on 2 Petri dishes with 15 cm diameter. Cells were incubated at 30 ^oC for at least 3 days.

2.2.2.3 Making spore plates and spore suspensions

Following the sporulation of transformant colonies, spores were harvested in order to make spore plates. For harvesting, 200 µl ST solution was pipetted on each colony and spored were gently scraped using an inoculation loop. After scraping, spores in ST were plated on a 9 cm Petri dish containing CM. Plates were incubated at 30 ^oC for 3 or 4 days.

Once the plates were covered with spores, plates were put in 4 ^oC for overnight maturation in order to make harvesting easier. Next day, 5 ml ST was pipetted on spores in the plate and spores were scraped from the medium surface by a Drigalski spatula. The solution was then collected, vortexed and filtered over nylon gauze. The filtrate was centrifuged at 5000 rpm for 10 minutes, supernatant was discarded. Spore pellet was re-suspended in ST and a diluted sample was taken to determine the concentration.

Counting was made under microscope by means of a hemocytometer. Spore suspension was then stored at 4 ^{o}C for further inoculation of cultures or making new spore plates.

2.2.3 Confirmation of transformation by PCR

2.2.3.1 Isolation of genomic DNA from *Aspergillus niger*

MM-Agar plates were inoculated with spores of transformants and grown overnight at 30 ^{o}C. Next day, 10-20 mg mycelia without spores were collected from medium by forceps and ground in a mortar with liquid nitrogen. Powdered cells were transferred to clean tubes and 100 µl extraction buffer, 7 µl 20 % SDS solution, 26 µl 5 M KAc buffer were added and incubated at 65 ^{o}C for 10 minutes. This incubation was followed by 10 minutes incubation on ice. Then the tubes were centrifuged at maximum speed for 10 minutes and supernatants were collected. 128 µl isopropanol and 12 µl NaoAC were added in each tube and tubes were incubated at -20 ^{o}C for at least 10 minutes. Next, tubes were centrifuged at maximum speed for 5 minutes and supernatants were discarded. Pellets were washed with 70 % ethanol, air dried and dissolved in 30 µl ddH_2O.

2.2.3.2 PCR

PCR was done using 0.1 µl and 0.5 µl of the isolated genomic DNA for each transformant. F_BTL2_S and R_BTL2_S primers were used. Reaction conditions were the same as described for bacterial colony PCR. Reactions were run on 1% agarose gel to see the products.

2.2.4 Activity screening

Rhodamine-containing plates were prepared according to the protocol by Kouker, G. and Jaeger K. E. (1987) Specific and sensitive plate assay for bacterial lipases.

Spore solutions of the transformants that gave positive PCR result was diluted to 10^3 spores/µl and 5 µl of spore solutions were spotted on Rhodamine plates. Plates were incubated at 30 ^oC for approximately 36 hours and screened under UV light.

2.2.5 Shake-flask cultures

Transformants that seemed to have higher activity than the wild type strain were selected for shake-flask cultures. Minimal medium was inoculated with 10^6 spores/ml. And the cultures were incubated in a 30 ^oC shaker at 250 rpm. Initial samples were taken and other samples were taken with approximately 12 hour intervals. Samples were centrifuged at 2500 rpm for 15 minutes at 4 ^oC, supernatant was transferred to a new tube. Cell pellet and supernatant samples were stored at -20 ^oC until further analysis.

2.2.6 Concentrating samples

500 µl of samples to be analyzed were concentrated in Speed Vac to a final volume of 50 µl.

2.2.7 SDS-PAGE Analysis

25 µl of concentrate samples were loaded on SDS gels. Protocol used for SDS-PAGE analysis was as given by Molecular Cloning: A Laboratory Manual, Sambrook et al. 2001. Gels were stained with Coomassie Brilliant Blue and Silver staining method.

2.2.8 Zymogram

SDS gel was incubated in 2.5 % Triton-X 100 for 30 minutes. Then it was transferred into 50 mM $NaPO_4$ buffer for 15 minutes. Then, 0.5 mM 4-MU-caprylate in 50 mM $NaPO_4$ buffer was poured on gel. Gel was incubated at room temperature for 5 minutes; photo was taken under UV light.

2.2.9 Activity Assay

96-well, black microtiter plates were used for activity assay. 20 µl of sample supernatants were added to wells. Then to each well, reaction mixture was added, containing 1 µl of 50 mM 4-MU-caprylate, 50 µl 0.4 M Tris-HCl, 129 µl ddH_2O. Assay was started immediately.

2.2.10 Computational Analysis

By means of an algorithm developed in our group, computational predictions of these heterologous expressions were done. Length of polypeptide, number of chains, molecular weight, pI, composition of amino acids in terms of hydrophobicity, charge, etc., composition of secondary structure elements, distribution of surface charges and surface area were given as input arguments and the protein was classified based on potential level of heterologous expression. Since a proper PDB file could not be obtained for ROL, predictions were made for BTL2 only.

3. RESULTS

3.1 Cloning of BTL2 and ROL genes into pAL85 vector

3.1.1 PCR

2 µl of PCRs done with F_BTL2_S/ROL_S and R_ROL_S were loaded on 1 % Agarose gel and run at 100 V. Gel photo was taken under UV (Figure 3.1).

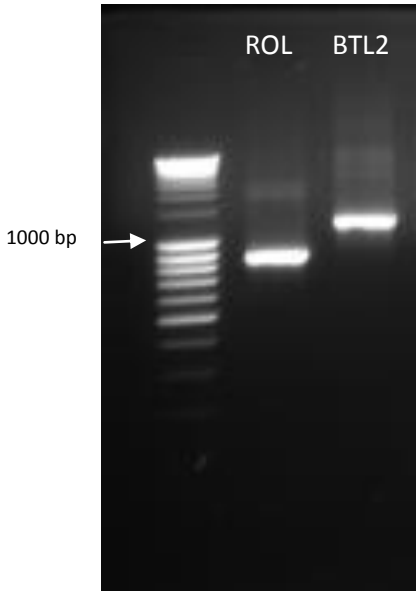

Figure 3-1: PCR products of ROL and BTL2

As indicated in Figure 3.1, PCR products were of expected sizes for ROL and BTL2. After this confirmation, whole PCR mixture was loaded on Agarose gel and the corresponding bands were isolated from gel by using Qiagen gel extraction kit.

3.1.2 Digestion

Digestion reaction of pAL85 with NdeI and NotI was run on 1 % Agarose gel. Corresponding band (Figure 3.2) was isolated from gel using Qiagen gel extraction kit.

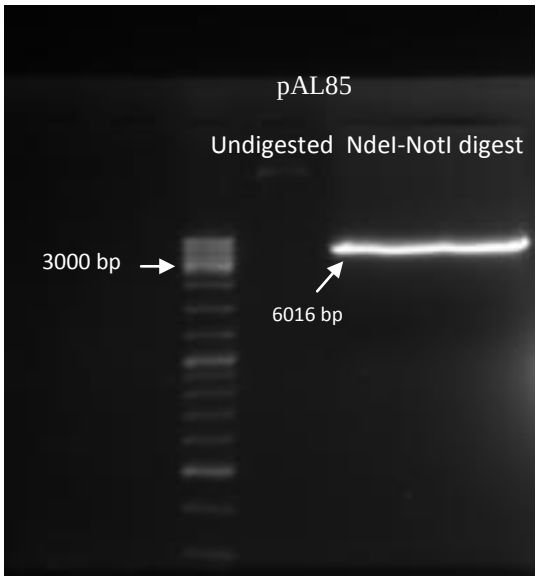

Figure 3-2: pAL85 digestion

3.1.3 Transformation

After ligation with in-fusion PCR cloning kit, competent E. coli XL-1 Blue cells were transformed with these reactions. Following transformation, 4 colonies were present on ROL plate, 3 colonies were present on BTL2 plate.

3.1.4 Colony PCR

Colony PCRs done with F_seq_pAL85 and R_seq_pAL85 were run on 1 % Agarose gel (Figure 3.3).

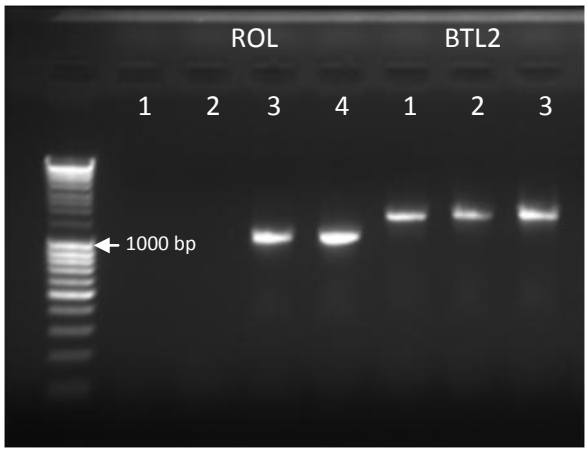

Figure 3-3: Colony PCR with sequencing primers

ROL-3, ROL-4, BTL2-1, BTL2-2, BTL2-3 colonies gave positive result. Liquid culture in LB-Kanamycin was started for these colonies.

3.1.5 Restriction and sequencing

Plasmids were isolated from liquid cultures by Qiagen Miniprep kit. Isolated plamisds were digested with NdeI and NotI. Digestion reactions were run on 1 % Agarose gel. (Figure 3.4)

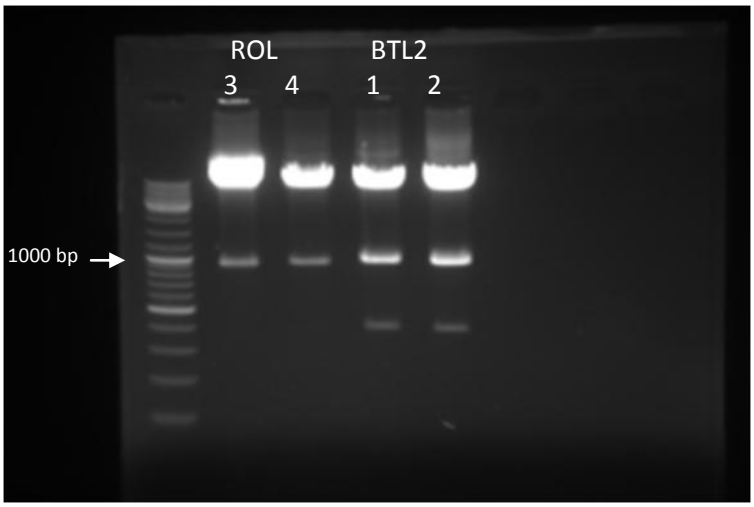

Figure 3-4: Confirmation before sequencing

Sizes of the bands seen in Figure 3.4, verified cloning reactions. Finally, these plamisds were sent for sequencing. Results from sequencing showed that ROL-4 and BTL2-2 were correct in sequence as well.

3.2 Transformation of *Aspergillus niger*

3.2.1 Transformation

In transformation plates, after 3 days of incubation, several colonies appeared. Plates that contained cells transformed with ROL gene had 10 colonies in total while BTL2-transformed cells formed 19 colonies.

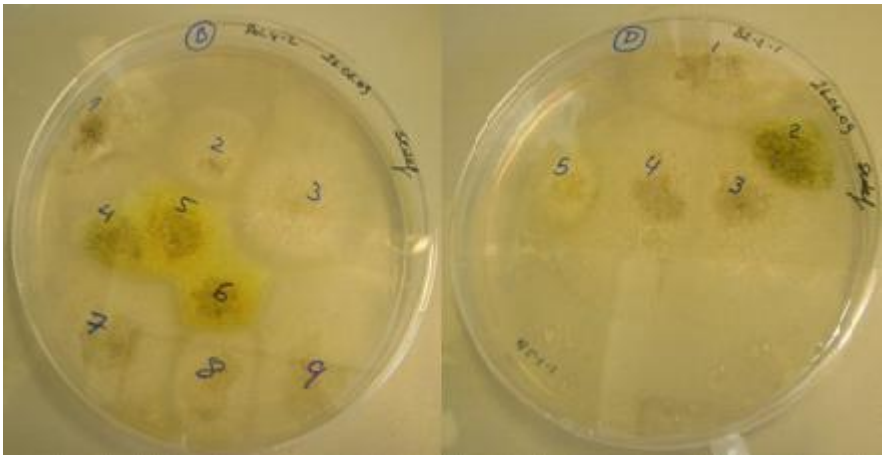

Figure 3-5: Transformation plates

3.2.2 Confirmation of transformation by PCR

A1, A2, A4, D1 and D5 colonies of BTL2 and B3, B7, C1, C8, C9, C10 and C11 colonies of ROL gene were selected for further analysis.

3.2.2.1 PCR

Results of genomic DNA PCRs done with sequencing and gene primers are given below:

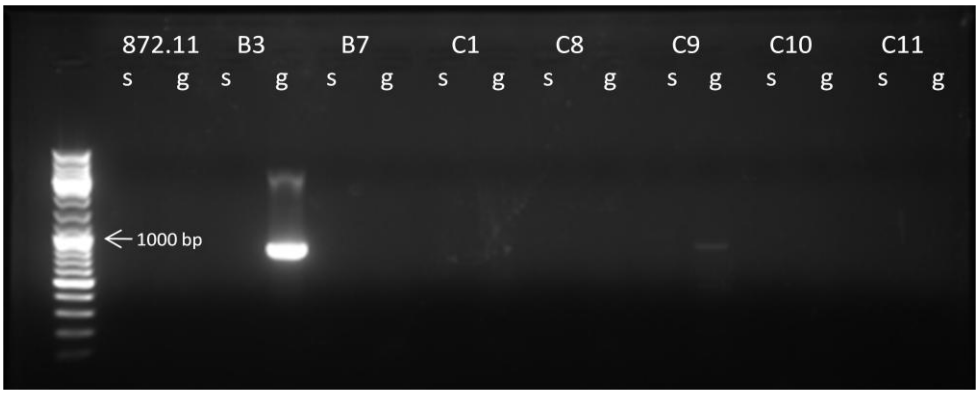

Figure 3-6: PCR from genomic DNA of ROL transformants

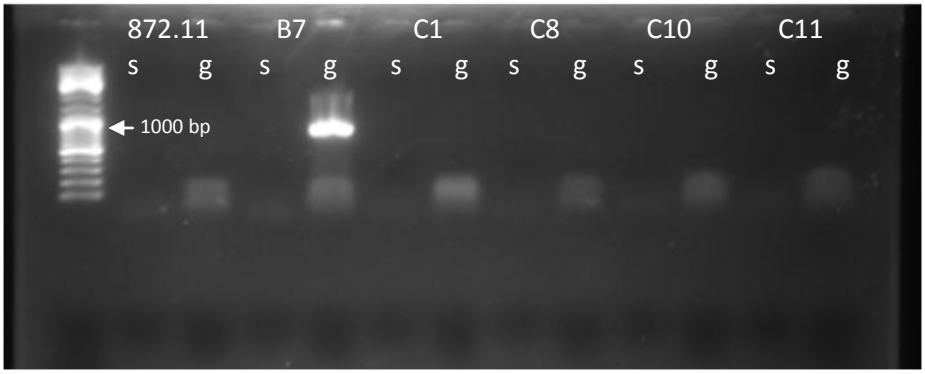

Figure 3-7: PCR from diluted genomic DNA of ROL transformants

Of the ROL transformants, B3, B7 and C9 gave the band of expected size with gene primers, therefore selected as positive strains.

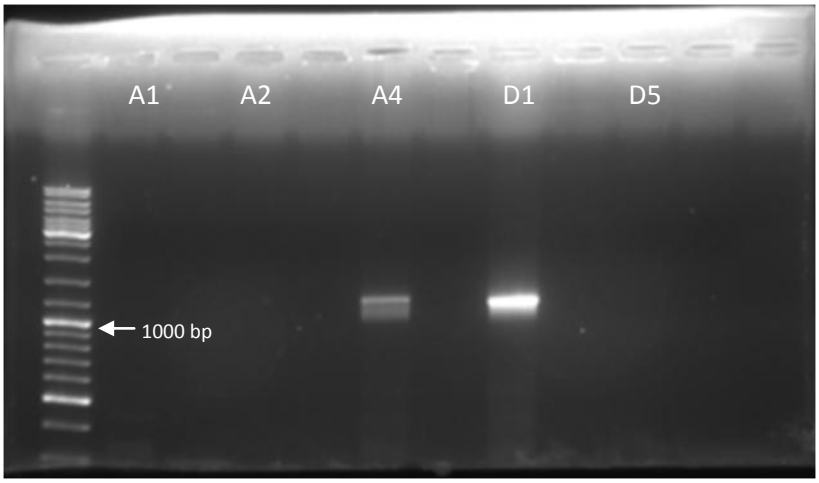

Figure 3-8: PCR from genomic DNA of BTL2 transformants

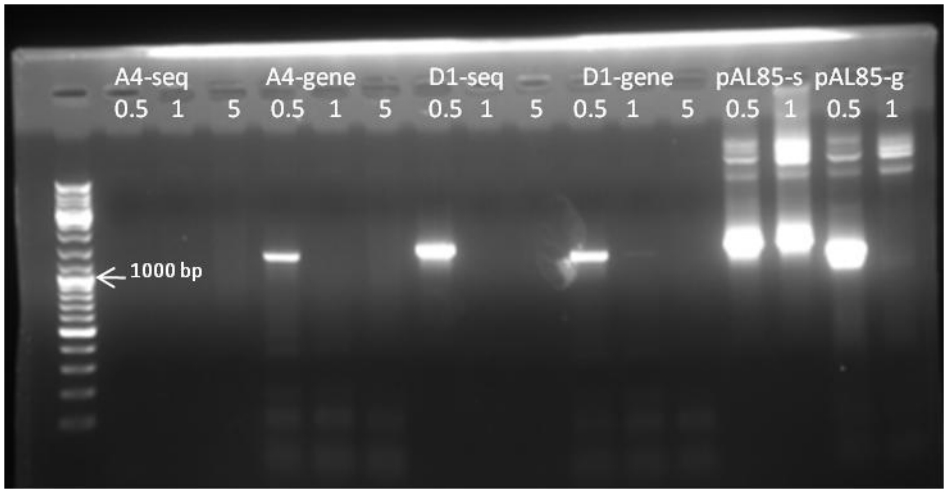

Figure 3-9: PCR from genomic DNA of A4 and D1

Among the transformants of BTL2 gene, A4 and D1 gave positive result for genomic DNA PCR. The plasmid pAL85 was also included in this agarose gel as a positive control to see if sequencing primers are working properly. PCRs of pAL85 gave correct result.

3.3 Activity screening

After 40 hours incubation of transformants on Rhodamine-containing medium, plates were screened under UV light. Images obtained are given below.

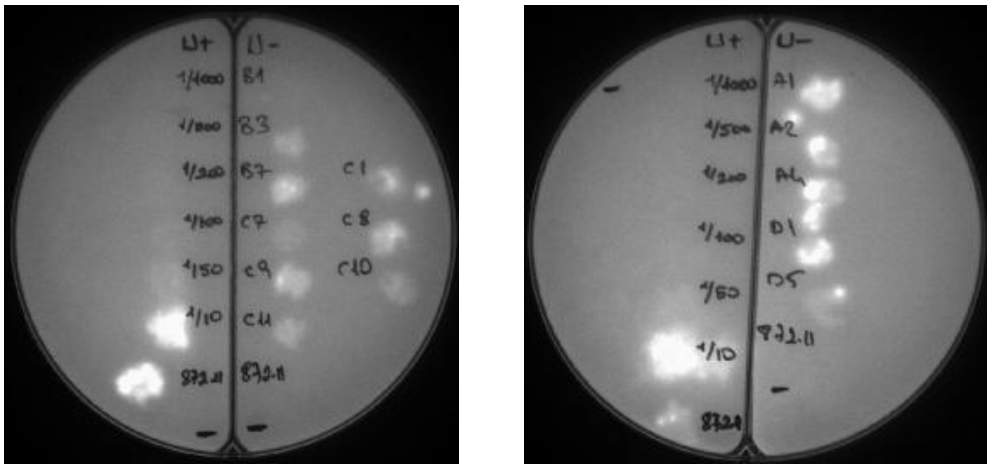

Figure 3-10: ROL (left) and BTL2 (right) transformants on Rhodamine plate

When ROL transformants were analyzed on Rhodamine plate, B3, B7 and C9 showed higher activity compared to other transformants. However, all transformants

showed lower activity and growth compared to the native strain. Based on the PCR data, B3, B7 and C9 were selected for shake flask cultures.

BTL2 transformants, except for D5, showed higher activity compared to the native strain. Since A1 showed high activity and A4 and D1 gave positive results for PCR, they were selected for shake-flask cultures.

3.4 Shake-flask cultures

3.4.1 BTL2 expression

3.4.1.1 SDS-PAGE

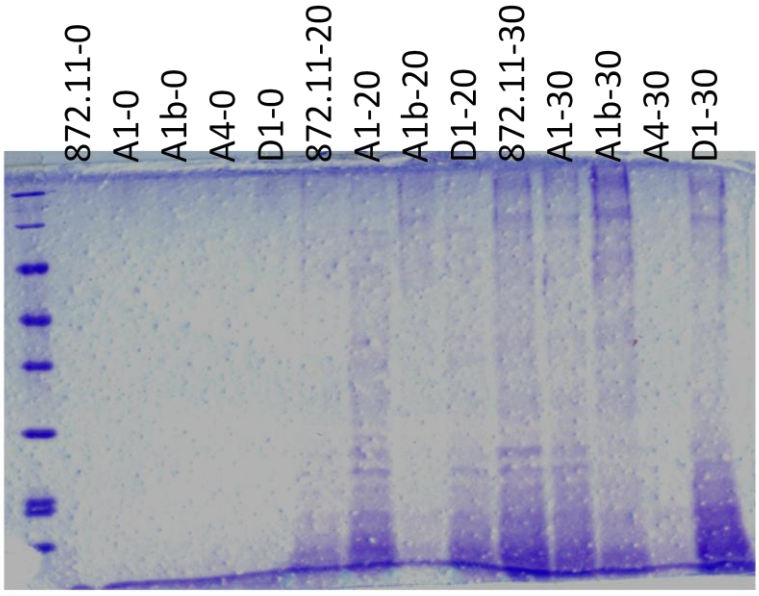

Figure 3-11: BTL 30-hour expression samples on SDS gel

Samples from first shake flask cultivation of A1, A4 and D1. A1b indicates use of baffled flask for A1 to investigate the effect of this condition on expression level. When the gel was stained, it was seen that A4 had the lowest amount of proteins while baffled A1 flask had the highest. D1 was also slightly higher than A1, in terms of thickness of bands on SDS gel. However, none of the strains showed a band of the expected size for BTL2 (43 kDa). SDS-PAGE results were also confirmed with Silver staining (data not shown).

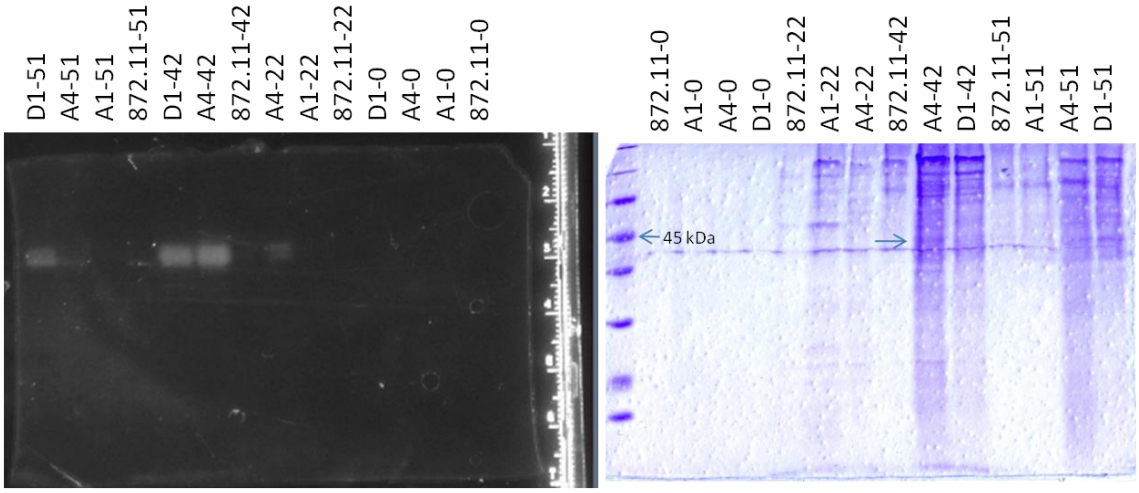

Figure 3-12: BTL2 51 hour expression samples

The next cultivation of BTL2 continued for 51 hours. Samples were again concentrated and run on SDS-gel. This time, zymogram assay was performed on gel before staining. Lanes corresponding to D1-51, A4-51, D1-42, A4-42, A4-22 showed activity. When stained, the corresponding locations of these bands were seen at expected size. Results were confirmed by Silver staining (data not shown).

3.4.1.2 Activity assay

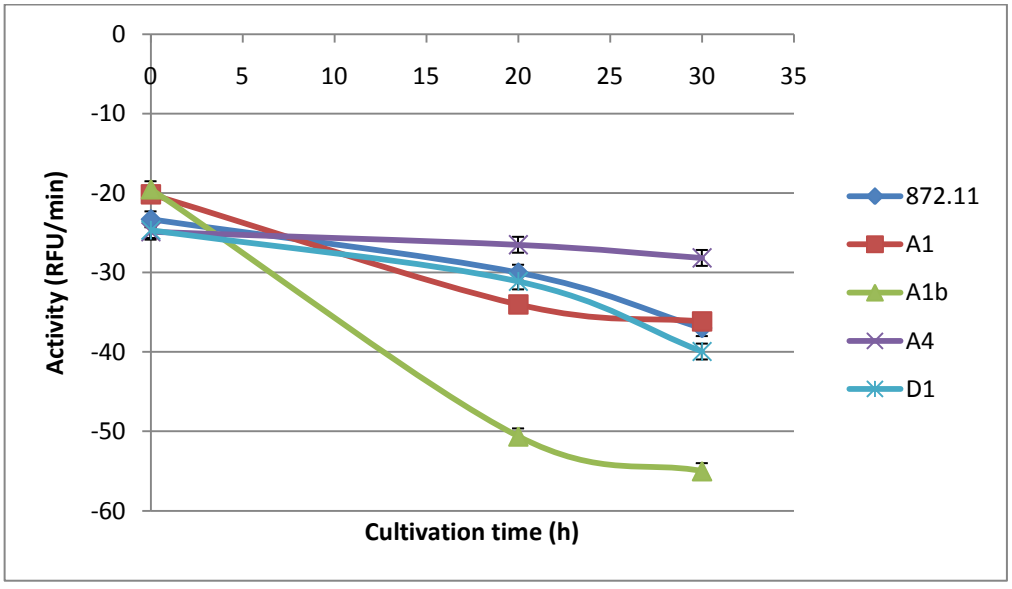

Figure 3-13: Activity assay of 30 hour BTL2 expression

Samples from 30 hours expression of BTL2 showed almost no lipolytic activity, parallel with the SDS-gel results.

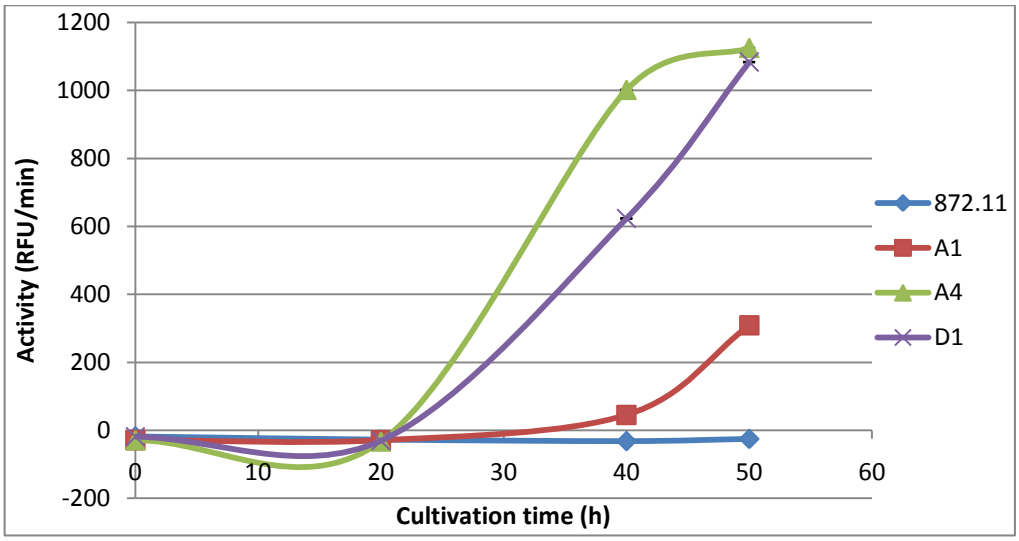

Figure 3-14: Activity assay of 51 hour BTL2 expression

Among the second expression samples, activity assay showed that the activity increased until the end of cultivation. Native strain showed the lowest activity while A4 was the highest. Until 40th hour, A1 had the same activity as the native strain, parallel to results of genomic DNA PCR and zymogram analysis. A4, on the other hand had the second highest activity.

3.4.1.3 Bradford assay

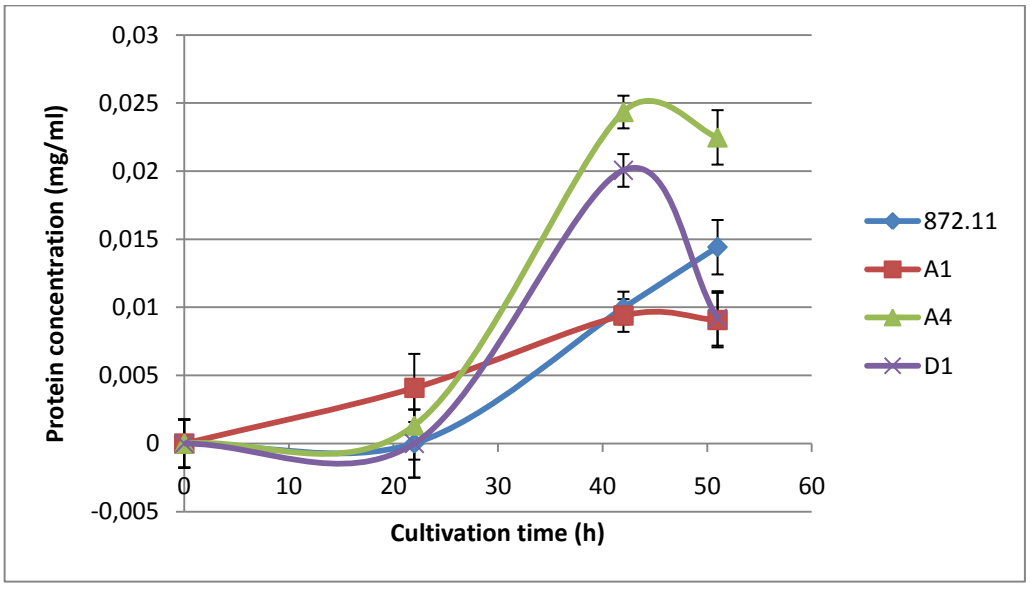

Figure 3-15: Bradford assay results for BTL2 expression

Bradford assay of BTL2 expression showed that strains A4, D1 and A1 reached their maximum protein concentration at nearly 42nd hour. The native strain, 872.11, on the other hand continued increasing until the end of cultivation, 51st hour. According to Bradford assay results, maximum protein levels for A4, A1, D1 and 872.11 were 0,024 mg/ml, 0,09 mg/ml, 0,009 mg/ml and 0,014 mg/ml, respectively.

3.4.2 ROL expression

3.4.2.1 SDS-PAGE

ROL expression samples were loaded on SDS gel after being concentrated. However, no bands were present corresponding to 29 kDa. Same results were obtained by Silver staining (data not shown).

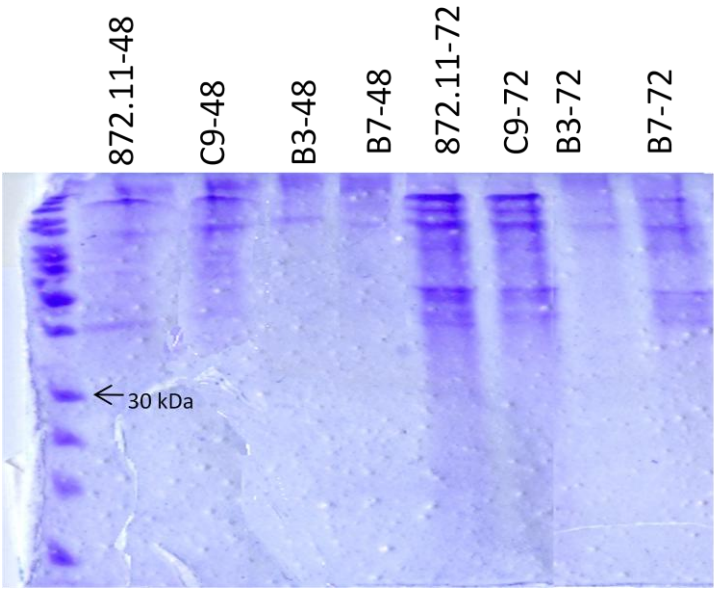

Figure 3-16: ROL expression samples on SDS gel

3.4.2.2 Activity assay

Results of the activity assays done against 4-methylumbelliferyl-caprylate are given below.

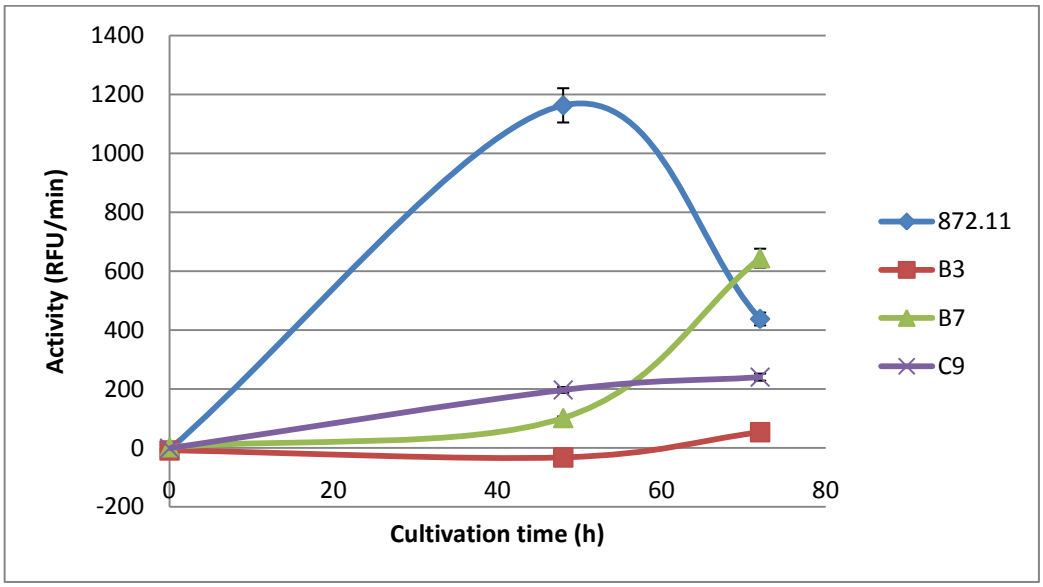

Figure 3-17: Activity assay of ROL expression

Native strain and C9 showed the same curve characteristics when the activities were graphed. 872.11 reached its maximum activity at nearly 48th hour with a value of 564 RFU/min. C9 also had its maximum activity close to 48th hour, with a value of 339 RFU/min. Activity of both of these strains started to decrease after 48th hour. B3 and B7, on the other hand, started increasing later than 872.11 and C9. B7 started to increase before 40th hour and kept increasing activity until the end of cultivation. Its final value was 618 RFU/min. B3 showed the most delayed pattern among these strains. Activity of B3 samples started increasing after 48th hour and slowly increased until the end of cultivation at 72nd hour. Its maximum activity reached was 71 RFU/min.

3.4.2.3 Bradford assay

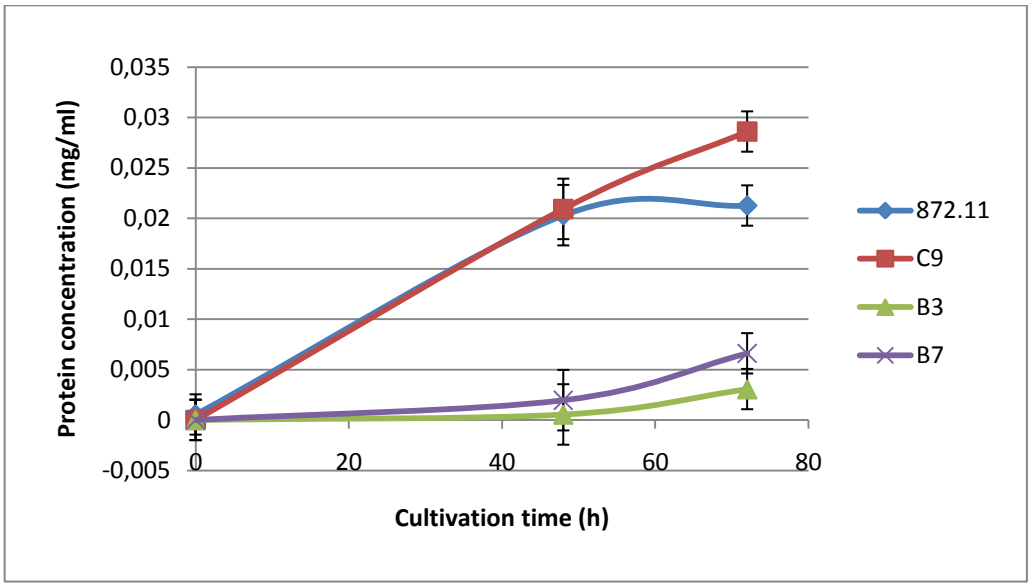

Figure 3-18: Bradford assay results for ROL expression

Among the analyzed samples, C9 had the highest protein concentration. It reached 0,028 mg/ml protein concentration at the end of cultivation. 872.11 had the second highest protein concentration. At the end of cultivation it had 0,02 mg/ml protein concentration. Strains B3 and B7 had 0,003 mg/ml and 0,006 mg/ml protein respectively.

3.5 Computational Analysis

Results of computational analysis showed that BTL2 protein belonged to the lowest expression class. At first calculation, two classes were created with the threshold between them being 1122 nM and BTL2 was in the lower group. Then, a second calculation grouped the data into three classes with thresholds 807 and 1631 nM. This time, BTL2 was included in the lowest class (less than 807 nM). Lastly, data was grouped into 4 classes with thresholds 684, 1122 and 1904 nM. Again, BTL2 was classified in the lowest (less than 684 nM) among 4 classes.

4. DISCUSSION

4.1. Construction of plasmids

pAL85 plasmid contained pkiA promoter which is a constitutive promoter, activated independent of the carbon source in the medium, which was favorable for an expression study. However, there was no signal sequence for the protein of interest to be secreted out of the cell. Therefore, 54 bp-long glucoamylaseA secretion signal was fused downstream of the promoter region by a series of PCRs, as described in Materials and Methods.

Multiple cloning site of pAL85 contained NdeI and NotI sites. For this reason, primers were designed so that the final product would have NdeI site on 5' end, NotI on 3' end. However, BTL2 gene was carrying a NotI site in its sequence. This was why pAL85-BTL2 gave three fragments when digested with NdeI and NotI. Since this would obstruct cloning via digestion and ligation, in-fusion cloning kit was used. The system used by this kit digested only from the ends of the fragments, leaving the other NotI site, thus the gene, intact.

4.2. Transformation of *A. niger*

After transformation, some of the colonies on plates were yellow while others were white. A previous research elucidated that the yellow color was caused by Aurasperone B, a secondary metabolite of the fungus (Nielsen, Mogensen, Johansen, Larsen, & Frisvad, 2009). During shake flask cultures, the same color was observed.

4.3 Confirmation of transformation by PCR

Among ROL transformants, only B3 gave positive result after the first PCR. The samples were five times diluted and PCR was repeated, using different amounts of template DNA. This time B7 and C9 also gave positive result. This means that the initial amount of template DNA was too much that inhibited the PCR. In case of BTL2 transformants, A4 and D1 gave positive result. However, for both ROL and BTL2 genes, sequencing primers did not work except for D1. For this reason, pAL85 vector was included as a positive control and together with D1, it showed that sequencing primers were working properly. The reason why no products were obtained when PCR was done with sequencing primers on B3, B7, C9 and A4 could be that the primers were binding to pkiA and trpC sequences in the genome, causing no products to be produced.

The native strain, 872.11 gave negative results in all PCRs. This result was expected because for gene primers, the strain did not have the target sequence in its genome and for the sequencing primers, pkiA and trpC regions were located apart.

4.4 Activity screening

When spores of ROL-transformed strains were spotted on Rhodamine-containing plates, native strain showed higher activity than all transformants but it had also grown faster. Even though none of them shoed higher activity than the native strain, based on the genomic DNA PCR results, positive strains were selected for shake flask cultures.

BTL2 transformants grew nearly the same as native strain but A4, D1 and A1 showed higher activity than the native strain. Therefore, these strains were selected for expression studies.

The fact that native strains showed activity on Rhodamine plates indicates that lipases of *A. niger* were also active. Accordingly, further analyses of transformed strains were compared to native strain to interpret the difference.

4.5 Shake flask cultures and analysis

To provide better aeration conditions, ratio of medium volume to flask volume was maximum 1/5 during cultivation. Also, to test the effect of baffled flask (McDaniel, Bailey, & Zimmerli, 1965), A1 was grown in both baffled and normal flask. As seen in Figure 3.12, A1b (baffled) has thicker bands compared to A1. Based on this finding, baffled flasks were used for following cultivations.

When SDS gel of the first BTL2 expression was analyzed, no bands were present at expected size. Moreover, A4 did not have any other bands at the end of 30 hours. This suggested that the cultivation could be held longer. Besides, the fact that the protein amount of these samples could not be detected by Bradford assay also proved that a very low level of protein was present. Finally, activity assay against 4-methylumbelliferyl yielded no activity for all strains, supporting that a very low amount of protein is present in the samples.

Following these data, next shake flask cultures of BTL2 transformants were done in baffled flasks and held for 51 hours. Samples were taken at 0, 22, 42, 51 hour time points. 500 µl of samples were concentrated to 50 µl and 25 µl of this sample was run on SDS gel. Zymogram assay that was performed before staining showed that samples A4-22, A4-42, D1-42 and D1-51 had activity. After staining, the bands that had activity were found out to be of the expected size for BTL2.

Activity assay also confirmed the results of zymogram analysis. A4 and D1 showed highest activity while the native strain had the lowest activity. According to Bradford assay data, A4 and D1 have maximum amount of protein in the samples, while A1 is the lowest. Since results in all analysis (zymogram, activity assay, Bradford assay) were positive for A4 and D1 but negative for 872.11, it can be concluded that BTL2 was expressed by strains A4 and D1. However, these results were not reproducible. In repeating experiments, zymogram and SDS gels did not give the desired result while activity could still be detected.

After 72 hours cultivation of ROL transformants, samples were taken and prepared for analysis as described previously. The samples were loaded on SDS-gel, but zymogram assay was not applied because it is known from previous studies in our lab that activity of ROL cannot be detected via zymogram since the enzyme cannot refold.

Therefore, the gel was stained directly. However, there were no bands present at around 29 kDa size in any of the lanes. Samples that belonged to B3 of every time point had the lowest amount of bands. This was expected because during cultivation, B3 flask was the one that grew least. 872.11 and C9 grew faster and in a similar fashion to each other whereas B3 and B7 were slower. Activity assay of these strains also indicated that activity of 872.11 and C9 started to increase immediately whereas B7 started at 40^{th} hour and B3 started at 60^{th} hour. Bradford assay confirmed these findings as well. Protein concentration of B7 started to increase at 20^{th} hour and B3 at 40^{th} hour. 872.11 and C9 on the other hand, started increasing from the initial time point. All analysis done with ROL transformants showed that strains B3 and B7 grow delayed compared to other strains. C9 was another strain that had the gene inserted in the genome but it did not express it.

4.6 Computational analysis

The classification made by the corresponding algorithm showed that these genes were not suitable for heterologous expression in *A. niger*. Since no bands could be obtained on SDS-gel for ROL and results for BTL2 were irreproducible, these data are consistent with experimental data.

4.7 Codon usage

In heterologous expression studies, changing the sequence of the gene of interest according to codon usage of the host organism is a common strategy. Presence of codons that rarely exists in that host organism causes pauses during translation, and this pause in turn lead to degradation of mRNA. Even if the translation is completed, some errors like frame shifts, amino acid deletions and reduced amount of protein synthesis occur (Kane, 1995). Thus, codon usage preferences of the host organism can cause both degradation of mRNA and lack of gene product although these two results may occur independently as well (Koda, Bogaki, Minetoki, & Hirotsune, 2005). Two examples in filamentous fungi are, optimization of potato α-glucan phosphorylase in *A. niger* (Koda,

et al., 2005) and mite allergen Der f 7 in *A. oryzae* (Tokuoka, et al., 2008). In both cases, mRNA and protein level increased significantly.

Based on this information, codon usage of *Rhizopus oryzae* and *Aspergillus niger* was compared, regarding the expression of ROL. Codon usage tables were obtained from Kazusa's Codon Usage Database (http://www.kazusa.or.jp/codon/). Using the data in the retrieved tables, first, the gene sequence of ROL was divided in 3-letter codons. Then, number of occurrence of each codon in ROL was counted and codons were ordered with respect to this parameter. Next, frequency of each codon of ROL in *A. niger* and *R. oryzae* was written from the table and the differences between were observed. Finally, number of occurrences of each codon in ROL was multiplied by the difference of frequency of that codon, to give a rough estimation of the effect on the translation. Minus (-) sign indicates a negative effect. Resulting table is given below:

Table 4.1: Comparison of codon usage of *R. oryzae* and *A. niger* for ROL

Codon	Aminoacid	Occurence in ROL	Freq in *R. oryzae*	Freq in *A. niger*	Difference	Estimated effect
GGU	G	17	36	17,5	18,5	-314,5
GUU	V	17	29,8	14,7	15,1	-256,7
AAG	K	14	19,9	29,6	-9,7	135,8
ACC	T	13	10,5	21,3	-10,8	140,4
CAA	Q	13	17,9	15,8	2,1	-27,3
UUC	F	12	10,8	5,1	5,7	-68,4
CCU	P	11	14,1	15,2	-1,1	12,1
GUC	V	11	26,4	21,8	4,6	-50,6
UCU	S	11	20,3	14	6,3	-69,3
AUC	I	10	38,5	6,9	31,6	-316
GAU	D	9	29	27,8	1,2	-10,8
AAC	N	8	16,1	21	-4,9	39,2
ACU	T	8	15,5	13,6	1,9	-15,2
CAU	H	8	14,4	12,3	2,1	-16,8
GCU	A	8	36,6	21,7	14,9	-119,2
GAA	E	7	35,8	24,8	11	-77
GCC	A	7	19	27,2	-8,2	57,4
UAC	Y	7	9	17,1	-8,1	56,7

UCC	S	7	10,4	19	-8,6	60,2
UUG	L	7	23,3	16,4	6,9	-48,3
AUU	I	6	44,3	22	22,3	-133,8
CUU	L	6	26,5	15,4	11,1	-66,6
CGU	R	5	7,8	10,1	-2,3	11,5
CUC	L	5	7,8	22,4	-14,6	73
UAU	Y	5	19	12,3	6,7	-33,5
UGU	C	5	10,1	5,8	4,3	-21,5
AGA	R	4	5,9	7,9	-2	8
AGU	S	4	10,9	10,6	0,3	-1,2
CAC	H	4	9,9	12,8	-2,9	11,6
GAC	D	4	14,6	27,2	-12,6	50,4
UUU	F	4	18,8	12,8	6	-24
AAU	N	3	18,9	14,9	4	-12
AGC	S	3	5,8	15,4	-9,6	28,8
CCC	P	3	8,5	17,8	-9,3	27,9
CUG	L	3	12	23	-11	33
GGC	G	3	13,3	22,5	-9,2	27,6
UGG	W	3	8,9	15,3	-6,4	19,2
ACA	T	2	16,2	12,4	3,8	-7,6
AUG	M	2	21,6	26,2	-4,6	9,2
GAG	E	2	24,5	34,7	-10,2	20,4
GCA	A	2	37	17,4	19,6	-39,2
GGA	G	2	16,6	16,1	0,5	-1
UGC	C	2	0,9	8,3	-7,4	14,8
AAA	K	1	36,9	14,1	22,8	-22,8
CCA	P	1	16,7	13,1	3,6	-3,6
CGA	R	1	8,1	9,3	-1,2	1,2
CUA	L	1	2	8,9	-6,9	6,9
GGG	G	1	12	12,6	-0,6	0,6
UCA	S	1	21,3	10,6	10,7	-10,7
UCG	S	1	5,5	14	-8,5	8,5

As the table indicates, most abundant codons in ROL are GGU and GUU which encode glycine and valine, respectively. When calculations mentioned above were done, however, neither glycine nor valine was found to have the highest estimated effect on translation. Codon AUC, coding isoleucine, with 10 occurrences in the sequence had the highest estimated effect due to the large difference of preference for this codon between *R. oryzae* and *A. niger*. When the table is investigated in terms of amino acids, isoleucine is found to be occurring 16 times in the protein and having the highest estimated negative effect on translation. To calculate the effect per codon, total estimated effect was divided by number of codons in ROL protein and the result was -3.10 which means that in total, use of a heterologous host has a negative effect for this case.

Even though BTL2 was shown to be expressed functionally, yield was lower compared to other expression systems, indicating that different strategies could be tried to increase efficiency.

It should also be noted that despite their ability to express and secrete proteins in high amounts, filamentous fungi are more difficult to handle in the laboratory, compared to yeast and bacteria, in terms of growth rate, morphology, culturing conditions, cell-based protocols etc.

5. CONCLUSION

Aim of this study was to explore potency of *Aspergillus niger* to produce ROL and BTL2.

Experiment results showed that BTL2 was being expressed by two of the transformants whereas ROL could not be detected. However, results obtained for BTL2 were not reproducible. But since activity could still be detected, experiment steps can be conducted again, under different conditions to find the required parameters for our aim.

Codon usage analysis showed that there are significant differences between *Aspergillus niger* and *Rhizopus oryzae.* Considering previous studies on codon optimization, we can suggest that optimizing codons of ROL for *A. niger* could increase the efficiency of this heterologous expression. Therefore, codon optimization and determination of mRNA level are regarded as future works.

When computational analysis was performed, it showed that BTL2 was not suitable for heterologous expression in *Aspergilus niger*; it was classified in the lowest expression class (less than 684 nM). These results also suggest that codon optimization might improve level of expression.

Finally, this study provided establishment of utilization of *Aspergillus niger* organism in our laboratory. Some materials, equipment and conditions were adapted for our laboratory. Even though this is a difficult process, the result is valuable since it enables a variety of new techniques and strategies to be carried out.

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7. APPENDIX

Media, buffers and solutions

Minimal Medium: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g, $MgSO_4$. 7 H_2O, 0.2 % casaminoacids, 0,1 % yeast extract dissolved in 1 L distilled water. pH adjusted to 6. Autoclaved. In case of solid medium, 15 g agar was added. Before use, 50 mM carbon source and appropriate supplements and 1 ml Vishniac trace-elements solution was added.

MMS-agar: Minimal medium salts and 325.2 g sucrose dissolved in distilled water. pH adjusted to 6. 3.6 g agar was added. Autoclaved.

Vishniac solution (Vishniac and Santer, 1957): 10 g EDTA, 4.4 g $ZnSO_4.7H_2O$, 1.0 g $MnCl_2.4H_2O$, 0.32 g $CoCl_2.6H_2O$, 0.32 g $CuSO_4.5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.47 g $CaCl_2.2H_2O$, 1.0 g $FeSO_4.7H_2O$ dissolved in 1 L distilled water. pH adjusted to 4 and autoclaved. Stored at 4^oC.

Saline-Tween solution: 0.9 % NaCl, 0.005 % (v/v) Tween-80 dissolved in water. Autoclaved.

Chemicals

2-Mercaptoethanol, Aldrich, Germany, M370-1

Agar, Merck, Germany, 101614

Coomassie Brilliant Blue, Merck, Germany, 115444

D-(+)-Glucose, Sigma, Germany, G-7021

Glycerol, Riedel -de Haen, Germany, 15523

K_2HPO_4, Merck, Germany, 105099

Kanamycin, Sigma, Germany, K4000.102

KH_2PO_4, Riedel –de Haen, Germany, 04243

L-Arginine, Sigma, Germany, A92600

Lyzing enzymes from *Trichoderma harzianum*, Sigma, Germany, L1412

NaCl, Riedel –de Haen, Germany, 13423

PEG 6000, Sigma, Germany, 81260

Rhodamine, Sigma, Germany, R6626

Triton X-100, AppliChem, Germant, A1388

Uridine, Sigma,Germany, U3750

Vitamin assay casamino acids, Difco, 228820

Yeast Extract, AppliChem, Germany, A1552

Equipment

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
Balance:	Sartorius BP211D, GERMANY
	Sartorius BP221S, GERMANY
Centrifuge:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
Deepfreeze:	-80 ^{o}C, ThermoForma, USA
	-20 ^{o}C, BOSCH, TURKEY
Electrophoresis:	Biorad Inc., USA
Gel Documentation:	Biorad, UV Transilluminator 2000, USA
Microwave Oven:	Bosch, TURKEY

Refrigerator: 4 ^{o}C, Bosch, TURKEY

Shaker: New Brunswick Sci., Innova 4330, USA

Forma Scientific, Orbital Shaker 4520, USA

Spectrophotometer: Schimadzu, UV1208, JAPAN

Spectrofluorimeter: Molecular Devices, SpectraMax Gemini XS

Speed Vacuum: Savant, Speed Vac Plus Sc100A, USA

Molecular Biology Kits

Qiaquick PCR Purification Kit

Qiaquick Gel Extraction Kit

Qiaquick Spin Miniprep Kit

Amresco Silver Staining Kit

Primers

Sense SS

Sequence (5'-3'):

ATGTCGTTCCGATCTCTACTCGCCCTGAGCGGCCTCGTCTGCACAGGGTTGG
CA

Length: 54

Anti-sense SS

Sequence (5'-3'):

TGCCAACCCTGTGCAGACGAGGCCGCTCAGGGCGAGTAGAGATCGGAACGA
CAT

Length: 54

F_ROL_S

Sequence (5'-3'): TGCACAGGGTTGGCATCTGATGGTGGTAAGGTTGTT

Length: 36

R_ROL_S

Sequence (5'-3'): AACTG ACAGCGGCCGCTCAATGATGATGATGATGATG

Length: 38

F_SS

Sequence (5'-3'): AATCATCCGTCCATATGTCGTTCCGATCTCTAC

Length: 33

pAL85_seq_F

Sequence (5'-3'): CCCATCTTCATCAATCC

Length: 17

pAL85_seq_R

Sequence (5'-3'): TGTATCTGGAAGAGGTAAAC

Length: 20

F_BTL2_S

Sequence (5'-3'): TGCACAGGGTTGGCAGCGGCATCCCCACGCG

Length: 31

Vector Maps

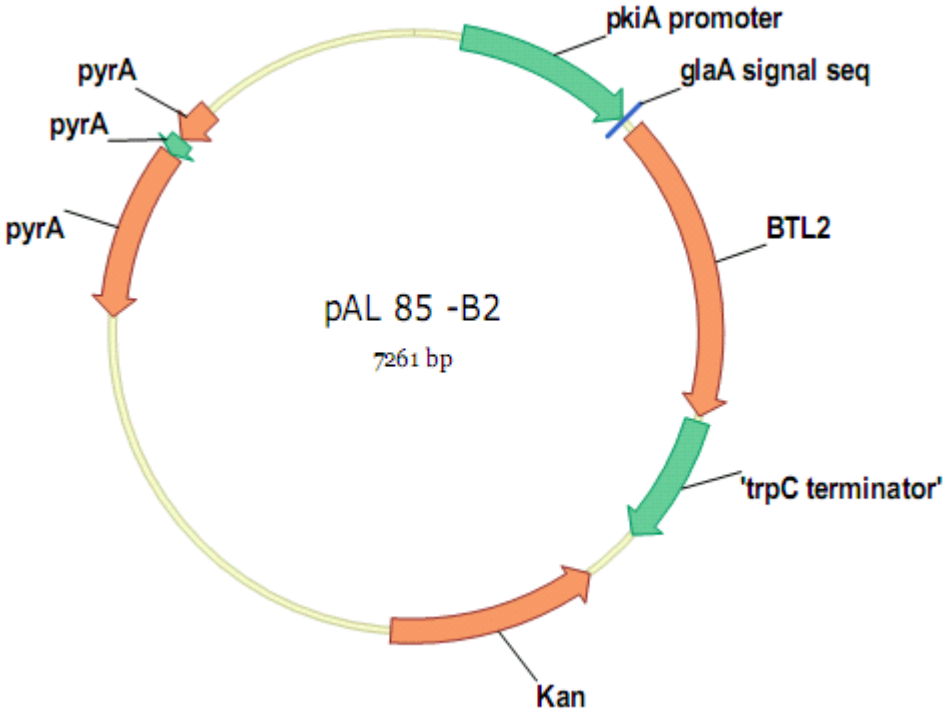

Figure 7-1: Map of pAL85-BTL2 construct

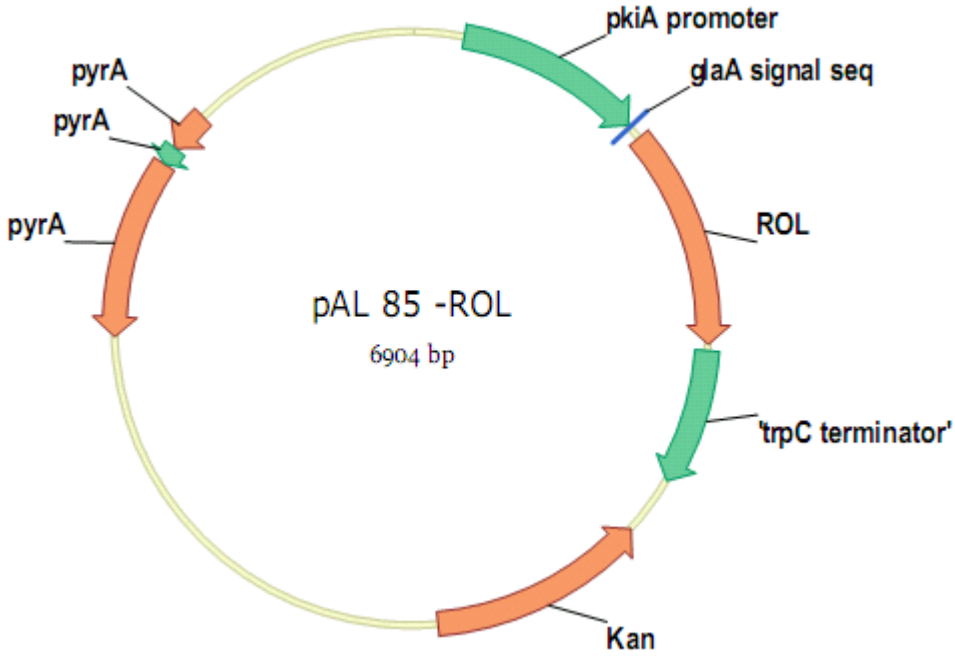

Figure 7-2: Map of pAL85-ROL construct