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STUDIES ON THE MICROBIAL DIVERSITY OF COMMERCIALLY IMPORTANT ENZYMES FOR ENANTIOSELECTIVE TRANSFORMATIONS ON A NEW RANGE OF 4-ARYL-1, 4-DIHYDROPYRIDINES

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ABSTRACT

A new series of 4-aryl-1, 4-dihyropyridines possessing potent calcium channel blocking activity along with good vasodilatory profile by the modified Hantzsch condensation using trifluoroacetic acid as catalyst has been synthesized and reported from our laboratory. The present work describes chemo enzymatic approach for the enantioselective hydrolysis of a 1, 4-dihydropyridine skeleton. In this study, lipases have been recognized as very useful biocatalyst. Diverse range of organisms including bacteria, fungi have been studied for extracellular enzyme production. Growth of the organisms and lipase production were measured at various intervals of time. Lipases from all the sources were assayed for at various temperatures ranging from 15 to 45ºC and pH in the range of 3.0 to 9.0. Variation of enzyme activity with substrate concentration has also been investigated. The lipases from above sources have been studied for their potential of enantioselective catalysis with the newly synthesized dihydropyridine diesters to obtain enantiopure products. Optical yields were confirmed with the help of polarimeter and HPLC analysis is being studied presently. The methodology for higher yields will be developed which could lead to production of optically active calcium channel blockers.

Key words: dihydropyridines, calcium channel blockers, chemoenzymatic synthesis, transformations

INTRODUCTION

Among the various calcium antagonists, dihydropyridines form the most important class of these compounds due to their high potency. Calcium antagonists have been introduced into clinical medicine over the last few decades. They interact specifically at one locus of cellular calcium regulation: the L-type calcium channel. In the absence of appropriate control mechanisms, calcium would be involved in events like cell destruction and death (Mannhold, 1994). Dihydropyridines belong to the class of nitrogen containing heterocycles having a six-membered ring (Fig. 1). This class of compounds shows fascinating spectra of pharmacological and therapeutic effects because of the heterogeneity involved in their chemical structure. 1, 4-Dihydropyridines exhibit a broad range of activities such as blocking of L-type calcium channels for the treatment of cardiovascular disorders, neuroprotective and antineurodegenerative, memory enhancing, antiviral and anti-inflammatory activities (Sobolev *et al.,* 2004). Many other properties displayed by 1,4-dihydropyridines include calcium channel modulation (Triggle, 2003), antidiabetic (Briede, 1999), geroprotective (Emanuel, 1985) and hepatoprotective (Bird,

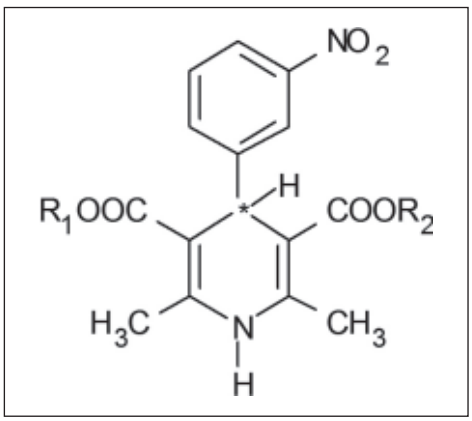

Fig. 1. Basic ring structure of dihydropyridines. R_1 and R_2 are different alkyl groups.

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1998) activities. In comparison to prototypical nifedipine type compounds, the second generation dihydropyridines possesses an asymmetrical ester substitution at positions 3- and 5- making these as chiral molecules. These asymmetric drugs not only differ in their action profile but also possess longer lasting activities, gradual onset of pharmacological effect and better tissue selectivity (Sobolev *et al.,* 2004).

It has been worked out that dihydropyridines with an unsymmetrical ester substitution are generally more potent than their symmetrical analogues e.g., nimodipine, manidipine and clinidipine (Goldmann *et al*., 1992a and 1992b). This observation has led to the development of numerous compounds with different ester substitutions at 3- and 5-positions. Variations in the phenyl substitution may include a nitro-group or an amino-group. The classical Hantzsch method used for the synthesis of dihydro-pyridines involves a one-pot condensation of an aldehyde with ethyl acetoacetate and ammonia for longer times producing racemic mixtures of unsymmetrical 1,4-dihydropyridines. One enantiomer may be responsible for the therapeutic effects of a drug whereas the other enantiomer is inactive and/or may contribute to undesirable and antagonistic effects. The use of single-enantiomer drugs can significantly lead to simpler and much more selective pharmacological effects, improved therapeutic indices and decreased drug interactions. Lipases have been found to be significantly suitable for enantioselective esterification, for derivation of novel compounds and resolving the enantiomers (Mustranta, 1992; Mertoli *et al.,* 1996).

For the preparation of enantiomerically pure 1,4-dihydropyridines very few reports are available whereas the demand for the chiral 1,4-dihydropyridine drugs has been increasing over the past few years. Although the current technologies for the asymmetric synthesis and chiral separation have shown some potential but there is still lack of viable and successful techniques that would produce single enantiomers in higher yields. Here in our study, stereo specific transformations by commercial lipases and by partially purified lipases obtained from bacterial and fungal sources have been carried out. The stages in this method involve fermentation to produce the required enzyme, partial purification of the enzyme, enzymatic transformation reaction and separation and purification of the products. Effective synthetic strategies need to be developed exhibiting superior control on stereo selective transformations of dihydropyridines, which can be replicated on larger scale as well.

MATERIALS AND METHODS

Materials

p-Nitrophenyl palmitate(pNPP) was procured from Lancaster Synthesis, England. The commercial grade lipolytic enzymes viz., *Candida rugosa* lipase (Sigma) and *triacyl glyceride* lipase (Hi-media) were used. The lyophilized stocks *Candida antarctica, Pseudomonas fluoroscens* and *Pseudomonas fragii* were procured from MTCC, Institute of Microbial Technology, Chandigarh. *Candida antarctica* was maintained and grown using Malt extract (3.0g/L), Yeast extract (3.0 g/L), Peptone (5.0 g/L) and glucose (10.0g/L) at pH of 7.0 at 25ºC with shaking for 76 hours. *Pseudomonas fluoroscens* and *Pseudomonas fragii* were maintained and grown on nutrient broth at a temperature of 30ºC and 25ºC respectively with shaking for 48 hours. Samples were collected at regular intervals. All the samples were subjected to turbidity measurements at a wavelength of 600 nm. Collected samples were centrifuged and supernatant collected referred as crude lipase (CL).

Assay of lipase activity

The extracellular lipase activity in the crude and purified lipase was assayed as described by Kanwar *et al.* (2005). The method made use of p-nitro phenyl palmitate as the substrate. Absorbance of p-nitro phenol released was measured at wavelength of 410 nm (Shimadzu UV/Visible Spectrophotometer, Japan). The unknown concentration of p-nitro phenol released was determined from standard curve of p-nitro phenol (2-20 µg/ml in 0.05 Tris buffer). One unit of lipase activity was defined as micromole(s) of p-nitro phenol released by hydrolysis of p-nitro phenyl palmitate by one ml of enzyme under assay conditions.

Lipase-catalyzed enantioselective hydrolysis

Recently, the synthesis of a new series of 4-aryl-1,4-dihyropyridines possessing potent calcium channel blocking activity along with good vasodilatory profile has been reported by Jain *et al.* (2006). The chemo enzymatic synthesis for the compounds with potent calcium channel blocking activity has also been proposed in the study. This type of enzymatic synthesis have been investigated with partially purified enzymes for initial screening. These investigations under different sets of conditions are desirable so as to optimize the reaction and to overcome some of the problems associated with the enzymatic reactions of dihydropyridine esters.

RESULTS AND DISCUSSION

Effects of parameters such as temperature and pH on lipase production have been reported by Sharma *et al.* (2011). Bacterial lipases are stable over a broad range of pH 4 to 11 as reported by Gupta *et al.* (2004). Our results indicate that lipases obtained from *Pseudomonas fluoroscens* and *Pseudomonas fragii* have the highest activity at pH of 8.5 (Fig. 2). On the other hand, pH optima of purified lipase (Hi Media) from *Triacyl glyceride* was found to be 8.0 and lipase from *Candida antarctica* (Sigma) was found be most active at a pH of 8.5 (Fig. 2). Our results indicate that the lipases under study here have pH optima in alkaline range.

Temperature optima of all the above lipases were also investigated. Lipase activity was determined at various temperatures of 15º, 25º, 37º and 45ºC (Fig. 3). Lipases obtained from *Pseudomonas fluoroscens*, *Pseudomonas fragii* and *Candida antarctica* showed increase in activity till the temperature of 45ºC whereas *Triacyl glyceride* lipase was found to be highly active at 25ºC. Further experiments need to be done to find the exact temperature maxima of lipase activity from different sources. It has been reported that bacterial lipases generally exhibit temperature optima in the range 30-60ºC (Litthauer *et al.,* 2002).

A new series of 4-aryl-1, 4-dihyropyridines synthesized in our laboratory are being used as substrates for the above enzymes to obtain enantiopure compounds. The enzymatic hydrolysis by purified lipase of *Candida rugosa* has been investigated using 1,4-dihyropyridine diester (Type-A) as substrate.

Enantioselective hydrolysis has been assessed in terms of optical rotations measured on Polarimeter (Autopol IV). Above studied compound (Type A) could be transformed to an enantiomer with a rotation of -12.72º (Fig. 4). These compounds subjected to enzymatic hydrolysis to produce optically active monoesters, were confirmed from a negative value of optical rotation. The complete composition for a test consisted of an enzymatic solution at a pH of 7.2 and the required concentration of the compound. For all test samples, two controls: enzyme control (EC) and substrate control (SC) were used which gave zero optical rotation as compared to tests those obtained a non-zero value of optical rotation. The non-zero (negative) values of optical rotation show that the enantioselective hydrolysis has been occurred. Further experiments are being done to study the various types of lipases so as to have the highest amount of the optically active compound. Effect of solvents, pH and temperature will be studied on the production of optically active compounds which have pharmacological activity. These test samples are being further analyzed using a chiral HPLC. Here in our study we are reporting that using dihydropyridines as substrates for microbial lipases, we are able to have stereo selective enzymatic transformations done. The study has the relevance and potential for production of enantiopure drugs with higher potency and reduced side effects.

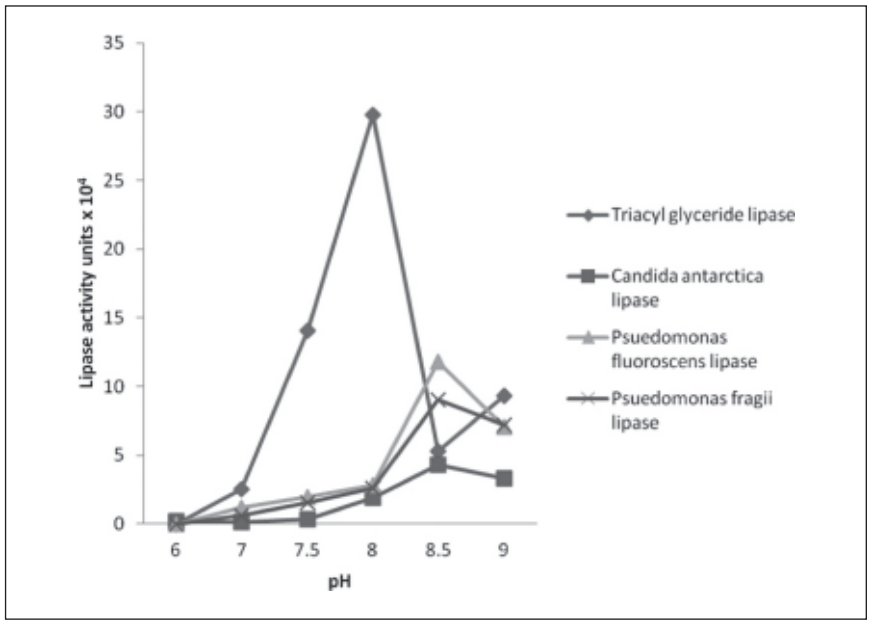

Fig. 2. Effect of varied pH on the activity of lipases from different sources as indicated.

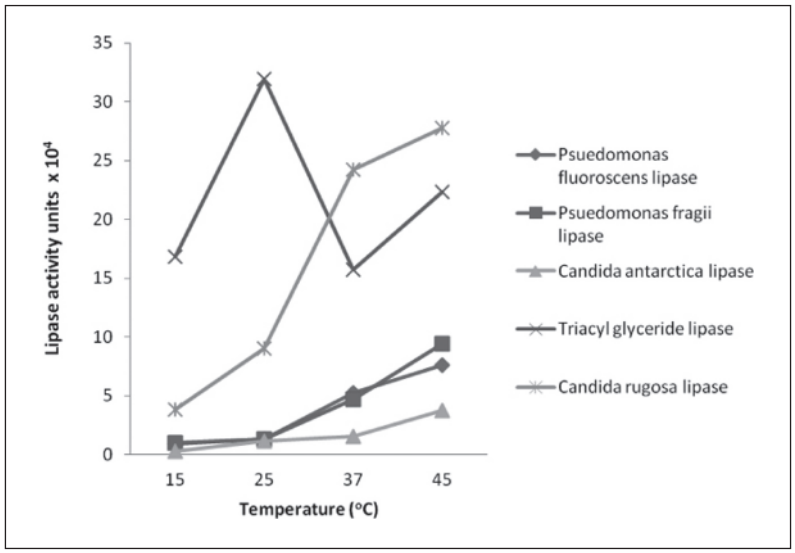

Fig. 3. Effect of temperature on the activity of lipases obtained from different sources as indicated.

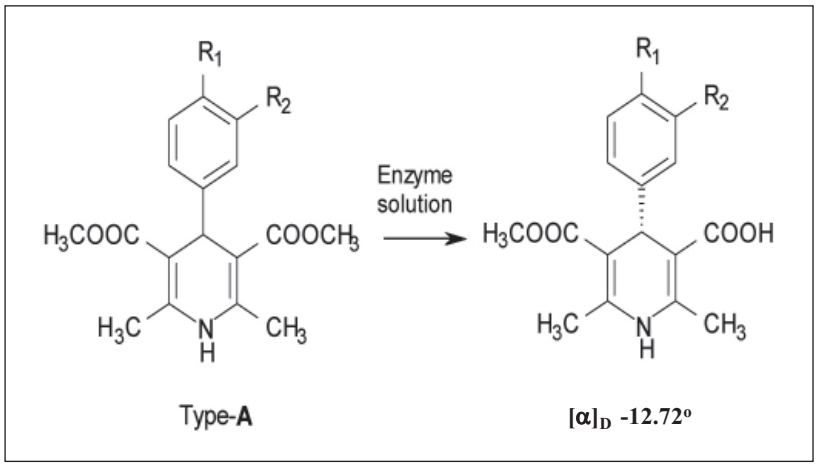

Fig. 4. Transformation of substrate in presence of enzyme to obtain the optically active compound.

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