Process and Enzyme Engineering of Aminotransferases for Improved Activity and Thermostability

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ABSTRACT

The production by biosynthesis of optically active amino acids and amines satisfies the pharmaceutical industry in its demand for chiral building blocks for the synthesis of various pharmaceuticals. Among several enzymatic methods that allow the synthesis of optically active aminoacids and amines, the use of aminotransferase is promising due to its broad substrate specificity and no requirement for external cofactor regeneration. The synthesis of chiral compounds by aminotransferases can be done either by asymmetric synthesis starting from keto acids or ketones, and by kinetic resolution starting from racemic aminoacids or amines. The asymmetric synthesis of substituted (S)-aminotetralin, an active pharmaceutical ingredient (API) has been shown to have two major factors that contribute to increasing the cost of production. These factors are the raw material cost of biocatalyst used to produce it and product loss during biomass separation. To minimize the cost contribution of biocatalyst and to minimize the loss of product, two routes have been chosen in this research: 1. to improve the engineering of the process by immobilization of biocatalyst in calcium alginate and addition of cosolvents 2. engineer the biocatalyst to have greater specific activity. The combined effect of immobilization and use of sodium dodecyl sulfate (SDS) as cosolvent allowed three successful recycles, but this recycling number was not significant enough to impact the process at multigram scale. As result of enzyme engineering an aminotransferase of higher activity was obtained. It would allow a reduction on product cost by reducing process time in 70% when used in the same concentration as its predecessors, or by decreasing enzyme concentration in a factor of 5 still achieving competitive yields.

INTRODUCTION

Optically active amines are an important class of organic compounds that can be widely used as useful chiral building blocks in asymmetric synthesis and medicinal chemistry (i). Some of the procedures currently available for synthesis of such compounds are the followings.

1. Hydrogenation of imines (ii)
2. Hydrogenation of oximes (iii)
3. Asymmetric borane reduction of oximes (iv)
4. Asymmetric hydrosilylation of imines or oximes (v)
5. Hydrogenation of enamides (vi)

6. Alkylation(vii)
7. Use of aminotransferases (viii)

Some of these procedures require expensive chiral catalysts or chiral reagents. Additionally some procedures are not stereoselective and as a result do not synthesize chiraly pure amine. The procedures mentioned above usually are done in several steps, in which costs are incurred due to reactors and purification of reaction mixture. Another approach to produce chiral amines is the use of aminotransferases, which have the advantage of producing the chiral amine in a single step without the need of chiral substrates. Such a process involves an amine donor compound (A), an amine acceptor (B), and an aminotransferase. The product (P) corresponds to a ketone with the same carbon structure as A, and another product (Q) corresponding to the amine with the same carbon structure as B. The reaction and reaction mechanism of transamination are shown in Equation (1) and Figure 1 respectively.

$$A + B \rightleftharpoons P + Q \qquad (1)$$

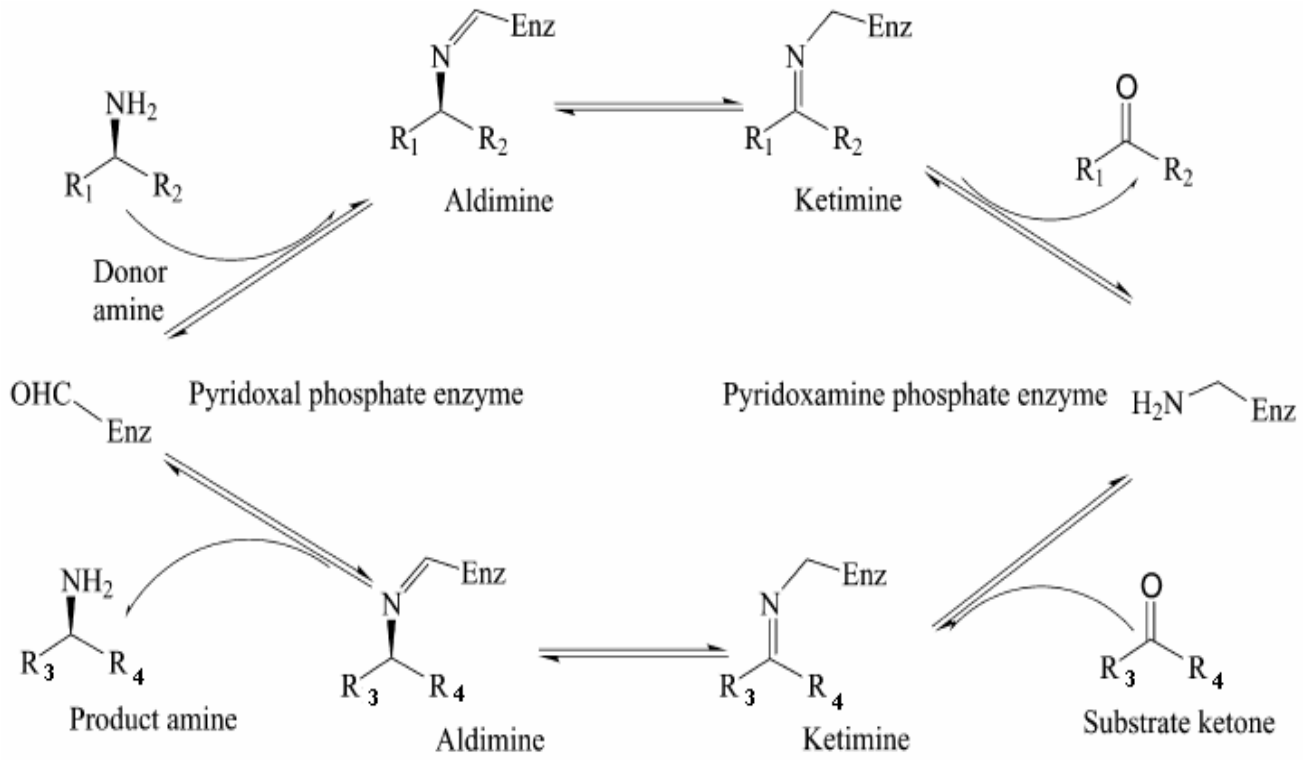


Figure 1: Reaction mechanism of Transamination (viii)

Such approach in the production of chiral amines is used by Cambrex North Brunswick, Inc, which specializes in the making of specific substituted (S)-aminotetralins, among other substances. The general description of this process at large scale is shown in Figure 2. During the production of substituted (S)-aminotetralins, depicted in the process described (Figure 2) there are costs associated with each step. Those costs have a large effect in the production cost of the chiral amine and they may be reduced. The key drivers for the overall cost of the product are:

1. Cost of the enzyme.
2. Concentration of product in the reaction system.
3. Process time.
4. Yield during product isolation.

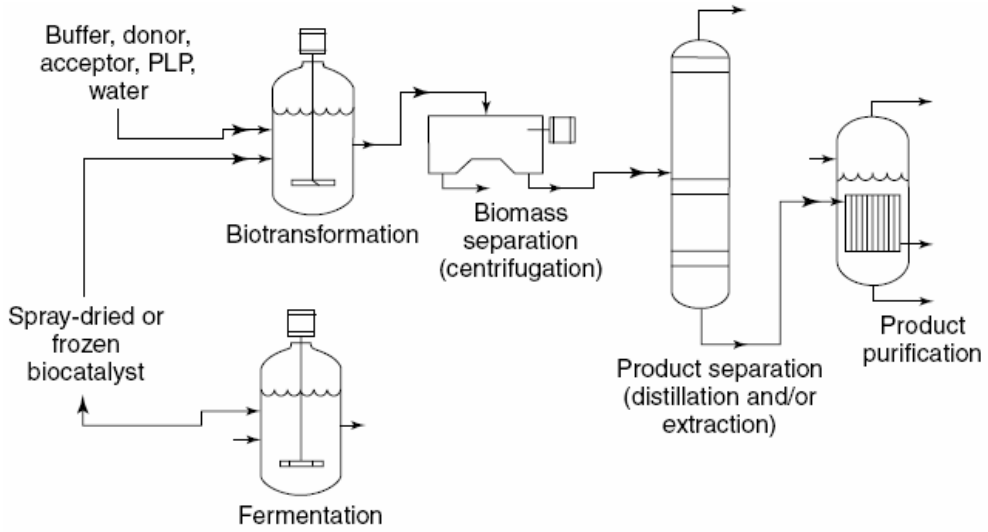


Figure 2: Biotransformation process for production of substituted (S)-aminotetralins (viii).

The reduction of the cost for producing substituted tetralone would be achieved when one of the following objectives or a combination of them is accomplished.

1. Reduction of enzyme concentration in the biotransformation process, which would be accomplished by an enzyme of higher activity than the current one (Mutant α).
2. Use of biocatalyst for several batches of production, which may be accomplished by immobilization providing that the biocatalyst is stable and has a long life time.
3. Increase of product concentration in the reaction system, which would be allowed by an enzyme that is active under altered conditions (temperature, substrate concentration).
4. Reduction of process time, which would be accomplished by an enzyme with a higher activity.

The research reported here addresses the above four objectives by generating and selecting a number of improved aminotransferases through random mutagenesis and screening.

Methodology

Molecular Biology

Bacterial strains and Plasmids: *E. coli* MG1655 (ATCC) was used for the production of recombinant aminotransferases. During intermediate steps in the selection of the best mutant, XL1-Blue MR Supercompetent Cells (Stratagene) were used as host cells. The aminotransferase gene (Stirling er al., 1992) was inserted into the multiple cloning site of the vector pSE420 (Invitrogen).

Error-Prone Polymerase Chain Reaction (PCR): Error-prone Polymerase Chain reaction was conducted using the GeneMorph^{TM} Mutagenesis Kit (Stratagene). This kit was

composed of Mutazyme® DNA polymerase, 10X Mutazyme® reaction buffer, 40 mM dNTP mixture, and 1.1 kb Gel standard. The reaction conditions were as follows: incubation at 95 °C for 5 minutes; 30 cycles of incubation at 96 °C for 30 seconds, 53 °C for 30 seconds, and 72 °C for 4 minutes. After the 30 cycles were completed, the reaction system was incubated at 72 °C for 10 minutes and finally the temperature was held at 4 °C. The reaction system was composed of 41.5 µl of RNA'se free water, 5 µl of 10X Mutazyme Buffer, 1 µl of 40 mM dNTP mixture, 0.5 µl of a mixture of Primer 1 and Primer 2 of concentration 250 ng/µl of each primer, 1 µl of Mutazyme DNA Polymerase, and 1 µl of plasmid template having a concentration between 10 pg/µl to 100 ng/µl.

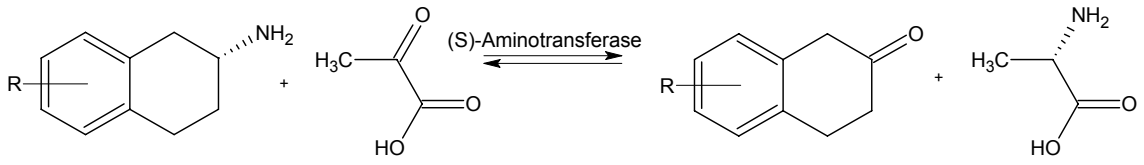
Restriction: The PCR product was purified using QIAquick PCR Purification Kit (Qiagen) and restricted using the enzymes HindIII (Promega) and NcoI (Promega). The restriction reaction was incubated for 1 hour at 37 °C using 9 µl of RNA'se free water, 4 µl of Buffer D (Promega), 1 µl of BSA (Promega), 25 µl of purified PCR product, and 1 µl of NcoI (Promega). The resulting product was purified using QIAquick PCR Purification Kit (Qiagen). This was followed by incubation for 1 hour at 37 °C using 9 µl of RNA'se free water, 4 µl of Buffer E (Promega), 1 µl of BSA (Promega), 25 µl of product of the previous step, and 1 µl of HindIII (Promega) followed by product purification using QIAquick PCR Purification Kit.

Ligation: Ligation was performed by mixing 20 ng of purified restricted PCR product, 20 ng of plasmid vector digested with HindIII and NcoI, 5 µl of 2X Rapid Ligation Buffer (Promega), 1 µl of T4 DNA Ligase (Promega), and nuclease free water to 10 µl. After 5 minutes at room temperature the reaction system was diluted by a factor of twenty.

*Transformatio*n: XL1-Blue MR Supercompetent Cells (Stratagene) were transformed with the mutant library following the manufacturer's protocol. Transformation into *E. coli* MG1655 was done after a treatment with magnesium chloride – calcium chloride.

Screening Procedure

The colonies obtained after transformation were propagated in triplicate onto LB Agar plates with 100 µg/ml of ampicillin (Sigma-Aldrich), and incubated for 9 hours at 37 °C. After overnight growth, the colonies were transferred to nitrocellulose paper. The nitrocellulose paper was placed on a filter paper soaked with the screening solution and incubated at temperatures between 50 to 80 °C. The selection of the mutant with improved properties was based on appearance of color. The substrates used in the assay were the substituted (S)-aminotetralin and sodium pyruvate (Figure 3). The reaction generated alanine and the substituted tetralone. The substituted tetralone upon exposure to air is a colored product.



Substituted (S)-Aminotetralin | Pyruvic acid | Substituted Tetralone (Colored product) | Alanine

Figure 3: Reaction for colorimetric screening

Immobilization

On a 100 ml basis, the proper amount of spray dried cells to achieve the desired concentration was mixed with 8 ml of craking solution. The composition of craking solution was 50 mM KH_2PO_4 (*Fisher Scientific*), 0.5 mM pyridoxal-5-phosphate, 1 mM dithiothreitol (*Sigma*) in distilled water. Afterwards, 92 ml of Sodium Alginate (*Fluka*) at a concentration of 3% (w/v) in distilled water was added. Finally 0.03 grams of pyridoxal-5-phosphate (*Schweizer Hall*), was added and mixed thoroughly. The mixture of spray-dried cells, cracking solution and sodium alginate was loaded to a pump and dropped through a needle -20(1/2) G- into 1000 ml of a solution of concentration 2% (w/v) [0.40 N] $CaCl_2$ (*J. T. Baker*) at room temperature, while gently agitating the solution for 2 hours. After harvesting the beads, they were washed with 1000 ml of distilled water.

Production of Substituted (S)-Aminotetralin

The production of substituted (S)-aminotetralin occurs upon reaction of isopropylamine (Sigma) and substituted tetralone (Cambrex) catalyzed by (S)-aminotransferase in the presence of pyridoxal-5-phosphate (Schweizer Hall), buffer and S-aminotransferase in immobilized or free form. The synthesis reaction is shown in Figure 4.

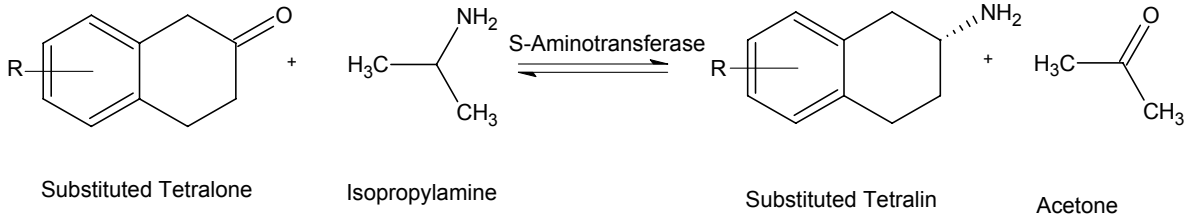


Figure 4: Reaction of substituted tetralone and isopropylamine catalyzed by S-aminotransferases to produce a substituted (S)-aminotetralin.

Biotransformation

500 mL of a solution containing 130 mM substituted tetralone (Cambrex), 750 mM Isopropylamine (Sigma), 2 mM Pyridoxal-5-Phophate (Schweizer Hall), and buffer (100 mM KH_2PO_4 (Fisher Scientific) or 200 mM sodium acetate(Sigma)) in distilled water at pH 7 was added to a 1,000 ml jacketed glass reactor (Wheaton). After adding the spray-dried biocatalyst, samples were taken periodically by withdrawing a sample (0.500 mL) and diluting it with 0.1 N HCl (9.5 mL) to stop the reaction. The progress of the biotransformation was determined by measuring substituted aminotetralin concentration by HPLC. All biotransformation reactions were carried out at optimum conditions of substrate concentrations, temperature, and pH for each mutant aminotransferase.

HPLC Analysis

Prior to analysis, the samples were incubated at 80 °C for 30 minutes in order to hydrolyze any by-product imine and then centrifuged to remove biomass. Samples were diluted and then analyzed using an HPLC (Hewlett-Packard Model 1100 Series). Absorbance readings were taken at 254 nm after injecting a volume of 15-25 µl. Flow rate was set to 1.0-1.5 ml/min for 20 minutes through a Nova-Pak Phenyl (Waters) column. The mobile phase was comprised of isopropanol: aq. buffer at 30%:70% (v/v), where the aqueous buffer was comprised of 0.24% (w/v) 1-Octanesulfonic acid (Janssen Chimica) and 0.15% (v/v) H_3PO_4 (85%w/w (Sigma)).

Results and Discussion

To achieve the research objectives, principles of process engineering were applied. Mutant α was immobilized (Figure 5). This mutant produce substituted (S)-aminotetralin at 50 °C and pH 7 in experiments where the immobilized enzyme was recycled. Initial rate for cycle 1 (6 hr duration) was determined, for cycle 2 (20 hr duration -1200 min-) it decreased by ~50% compared to cycle 1, and for cycle 3 (20 hr duration) it decreased by ~90% compared to cycle 1 (immobilized preparation consisted of 50 mg of spray dried cells per gram of calcium alginate). Total product accumulation for each cycle decreased as well, from 100% in cycle 1, 80% in cycle 2, and 30% after cycle 3.

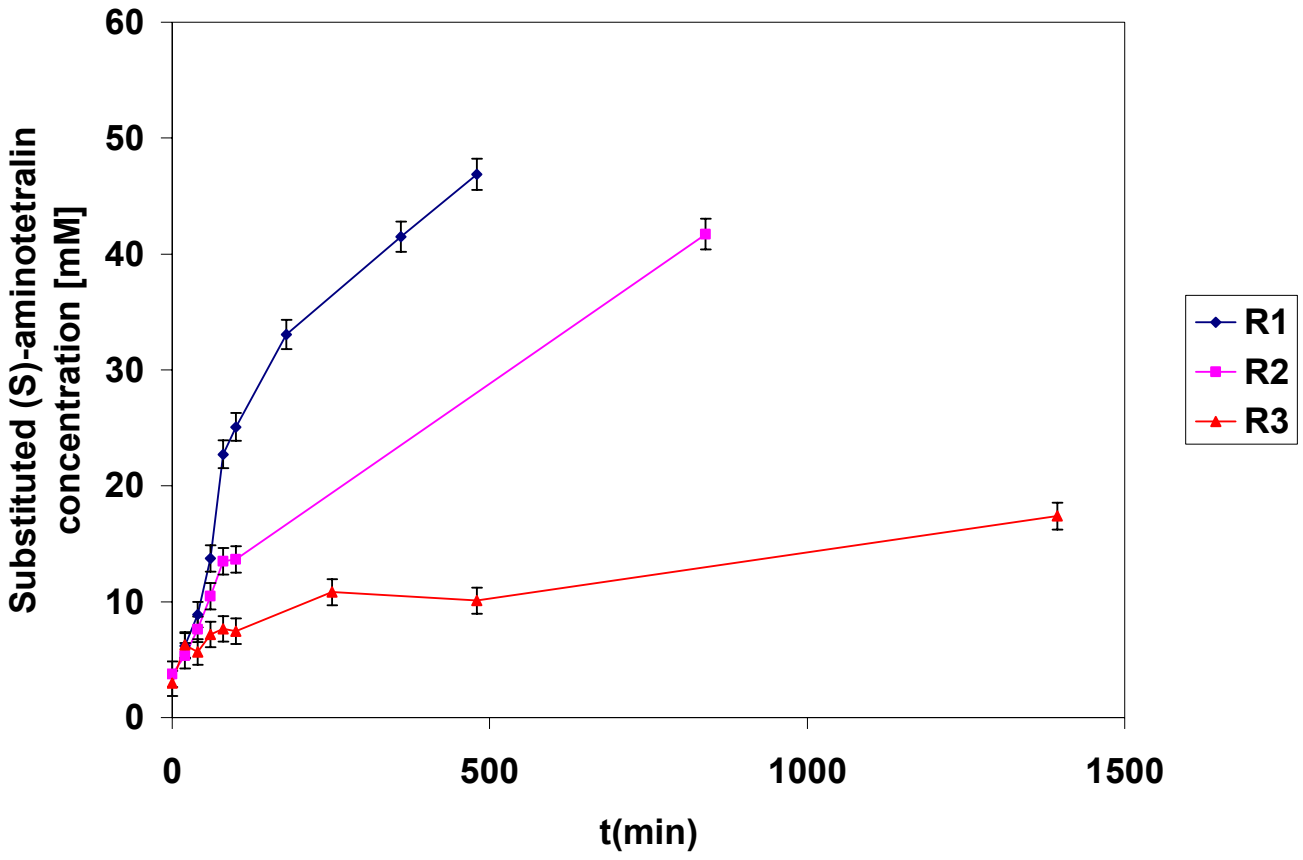


Figure 5: Production of substituted (S)-aminotetralin by immobilized Mutant α at 50 ºC, pH 7, Eo= 5 g/l.

This enzyme was determined to be deactivated at elevated temperatures during the reaction cycle and was not stable enough to allow multiple cycles in its immobilized form. In order to achieve the research goal using immobilization, it was necessary to engineer it to develop an enzyme with improved thermostability. Using as a template Mutant α, Mutant β was obtained. Mutant β was used together with another engineering approach, besides immobilization, the use of cosolvents. Since substituted tetralone has limited solubility (33 mM at 55 °C) a cosolvent would increase its solubility, which may increase activity (reaction rate). Toluene, and sodium dodecyl sulfate were tested as cosolvents, out of which sodium dodecyl sulfate was the best. Mutant β was immobilized and tested in a system with 0.01% (w/v) sodium dodecyl sulfate. SDS at 0.01% (w/v) allowed three recycles of the immobilized enzyme (Figure 6), always reaching higher product concentration than the system with toluene at 3% (v/v).

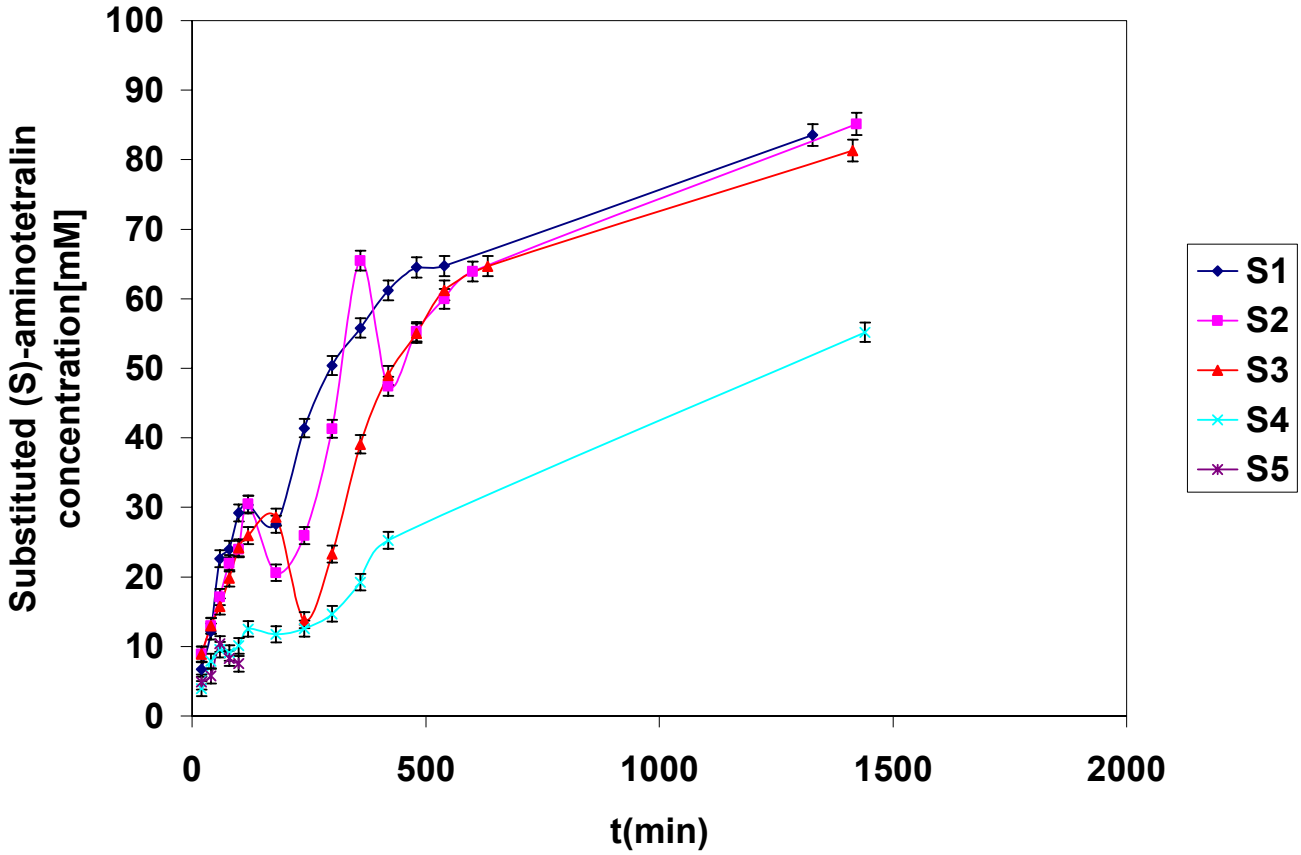


Figure 6: Production of substituted (S)-aminotetralin by immobilized Mutant β at 50 °C, pH 7, 0.01% (w/v) sodium dodecyl sulfate, Eo= 5 g/l.

The initial rate of immobilized enzyme in the system with SDS 0.01% (w/v) at 50 °C (Figure 6), pH 7 was retained for three cycles but dropped precipitously in the fourth cycle. The final product concentrations for each cycle also followed the same pattern. Although significant improvement of immobilized enzyme productivity and stability were observed by one round of mutagenesis; it was concluded that three recycles of the enzyme were not significant enough to impact on the production cost of the product. The decrease on activity in recycles 4 and 5 (Figure 6) was due to trapping or deposition of the desired product –in a concentration of 13%

w/w in the bead- and the substrate ketone -in a concentration of 2% w/w in the bead-. Since the immobilization approach did not seem to be a suitable solution to the problem, the strategy then to achieve the research goal aimed toward improving activity of the biocatalyst.

Using as template Mutant β, Mutant γ was obtained. Figure 7 shows its activity. Mutant γ at the same concentration in the reaction at which Mutant α and Mutant β were used allowed a 70% reduction in process time; from 24 hours (Figure 5 and Figure 6) to 6-7 hours (Figure 7). Also, Mutant γ at a concentration of 1 gram per liter in the reaction allowed competitive yields after 24 hours. The use of Mutant γ in a way in which it reduces process time or enzyme concentration accomplishes the research goals at some degree; since the production cost is a function of the cost of fixed raw materials, the amount of enzyme used, and process time.

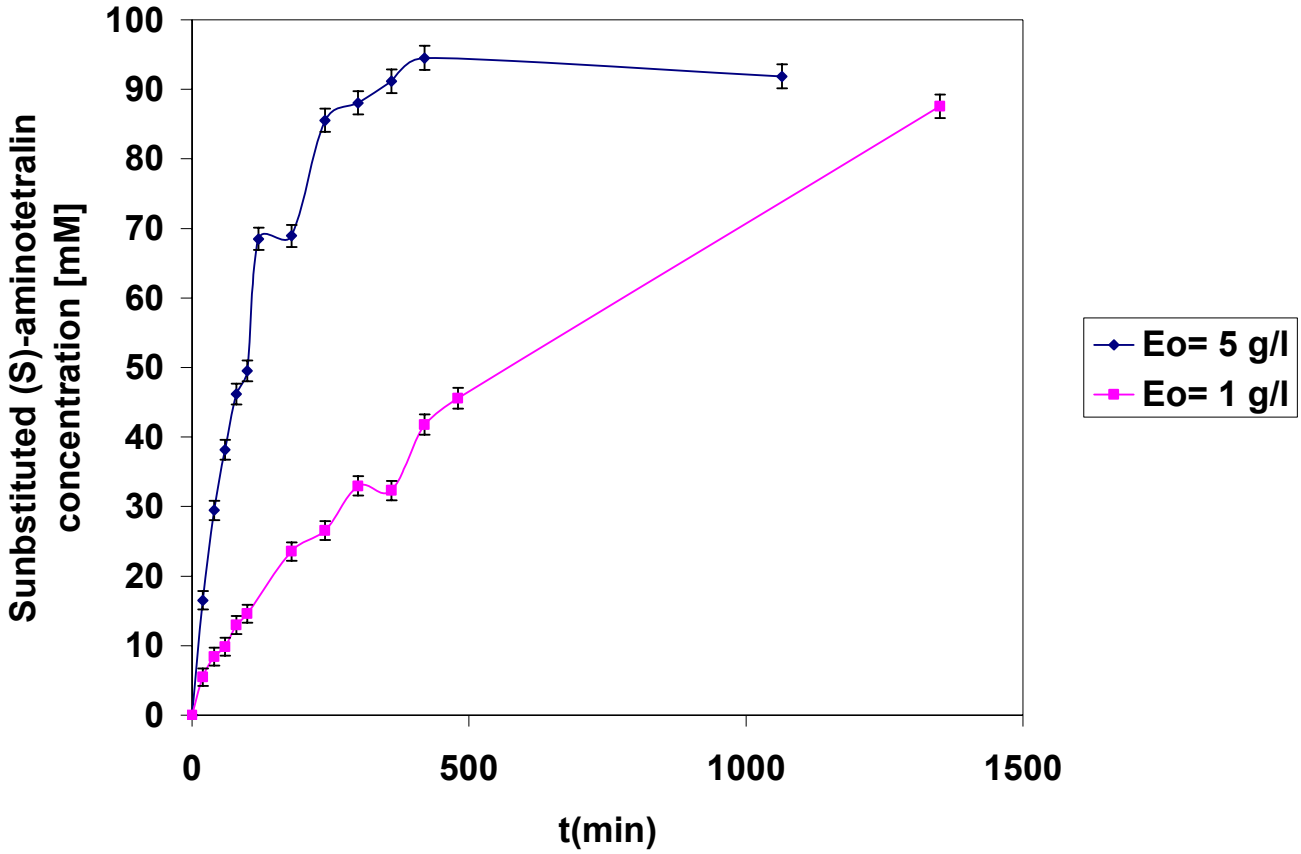


Figure 7: Production of substituted (S)-aminotetralin by free Mutant γ at 55 °C, pH 7, Eo= 5 g/l and 1 g/l.

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