

Improved Enantioselectivity of *Candida antarctica* A Lipase Through Sol-Gel Entrapment

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Abstract: Lipase from *Candida antarctica* A was immobilized for the first time by sol-gel entrapment. Two immobilization methods, with binary and ternary silane precursor mixtures containing tetramethoxysilane and trimethoxysilanes with alkyl or aryl groups and an ionic liquid as additive, have been applied. The catalytic activity and enantioselectivity of the immobilized biocatalysts were evaluated in the transesterification of aliphatic *sec*-alcohols with vinyl acetate. Depending on the sol-gel entrapment method applied, we obtained biocatalysts with excellent transesterification activity ($> 0.705 \mu\text{mole}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$) or with high enantiomeric ratio ($E > 110$) for the tested substrates. These results demonstrate the high biocatalytic potential of *Candida antarctica* A lipase and open new perspectives in future applications.

Keywords: *Candida antarctica* A lipase, sol-gel entrapment, acylation, enantioselectivity

1. Introduction

The increasing demand for enantiopure compounds led to significant progress in asymmetric synthesis and catalysis. A valuable strategy in biocatalysis was the exploitation of the natural "pool" of chiral molecules as source of catalysts. Between these chiral molecules, the use of enzymes, is advantageous because they are developed by nature, are biodegradable, able to work in mild conditions and usually present high chemo-, regio- and stereoselectivity, thus leading to cleaner reactions [1].

Among enzymes, lipases have been most frequently employed as catalysts for enantioselective transformations in organic synthesis [2-3]. They accept a broad range of substrates and often display good enantioselectivity, being particularly useful in the kinetic resolution (KR) of racemic alcohols and amines. The enzymatic KR can provide two separated enantiomers, with 50% theoretical maximum yield for each of them. The maximum yield can be improved up to 100% if the enzymatic KR is coupled with *in situ* racemization of the remained substrate, to transform all of the substrates to single enantiomeric products, called dynamic kinetic resolution (DKR) [2].

Two lipases isolated from *Candida antarctica*, lipases A and B, are among the most valuable biocatalysts. Lipase B (CALB) is one of the most frequently used enzymes for industrial applications nowadays. Lipase A (CALA) was reported to be a thermostable lipase, therefore could be interesting for industrial use. Furthermore, this enzyme was found to exhibit high activity towards sterically hindered alcohols, including both secondary and tertiary alcohols [4].

There are many available immobilization methods, including adsorption, covalent attachment to solid supports [5], cross-linking [6], but entrapment of enzymes in

inorganic/organic hybrid polymer matrices has received a lot of attention in recent years and has provided a particularly easy and effective way to immobilize purified enzymes [7-8]. In addition, the sol-gel process allows high compositional and morphological flexibility, by utilization of alkoxyde-type silane precursors holding one or two nonhydrolyzable organic functional groups and various additives, to yield organic-inorganic hybrid matrices [8-9].

The method of immobilization may considerably influence the properties of the biocatalysts [9-10]. In many cases immobilization is followed by a decrease in enzyme activity or a deterioration of other catalytic features. There are some reports in the literature that demonstrate how immobilization of an enzyme may also improve its activity, specificity and selectivity [9-13]. These improvements in the performance of enzymes after immobilization are not always consequences of a more active or selective enzyme molecule, but to some artifact which can alter the activity of the free or immobilized enzyme, or just affect its stability [13]. As concerns the influence of immobilization on enantioselectivity, Jacobsen *et al.* reported that the enantioselectivity of CALB for the kinetic resolution of racemic secondary alcohols was not changed by immobilization [14], but the main opinion is that immobilization may alter K_M or K_{cat} towards one or both enantiomers, leading to significant change of enantioselectivity [13].

Although CALA is an important lipase, the reports concerning its immobilization are scarce. Ondul *et al.* carried out CALA immobilization on cotton terry cloth fibrils coated with polyethyleneimine, by adsorption and cross-linking with 0.2% glutaraldehyde [15]. CALA adsorbed on Celite was an efficient biocatalyst for the hydrolysis of N-acylated 1-(2-phenylthiazol-4-yl)ethanamines, but the enantioselectivity of this reaction was lower compared to Novozyme 435 [3]. The most

important study was performed by Boros *et al.*, a systematic investigation of hydrophobic adsorption of CALA on functionalized silica supports. Despite the good catalytic activity, the enantioselectivity of these prepares remained low against 1-phenyl ethanol (E values below 2.0) [16]. Up to date, an immobilization method of CALA leading to a biocatalyst with high enantioselectivity in the kinetic resolution of secondary alcohols was not yet reported.

In this work, CALA was immobilized by sol-gel entrapment techniques, using two experimental protocols with binary and ternary silane precursors systems. The influence of the immobilization method and the nature of the silane precursors on the catalytic properties of immobilized lipases has been investigated. The acylation of aliphatic *sec*-alcohols by vinyl acetate was used as model reaction to evaluate the catalytic activity and selectivity.

2. Experimental

2.1. Materials

Candida antarctica A lipase was purchased from ChiralVision. Silane precursors methyl- (MeTMOS), octyl- (OcTMOS), vinyl- (VTMOS) and phenyl-trimethoxysilane (PhTMOS) were purchased from Merck and tetramethoxysilane (TMOS) from Fluka, Germany. Other materials used: tris-(hydroxymethyl)-aminoethane (Loba Chemie, Germany), 2-propanol (Merck, Germany), sodium fluoride and 2-hexanol from (Fluka, Germany), 2-heptanol (Merck), 2-octanol, vinyl acetate, *n*-hexane (98%), hydrochloric acid (37%) were of analytical grade and have been used as purchased from Merck, Germany. *n*-decane and *n*-dodecane (Sigma-Aldrich, Germany) were used as internal standards for quantitative gas-chromatographic analysis. The ionic liquid 1-octyl-3-methyl-imidazolium tetrafluoroborate ([Omim]BF₄) was product of Fluka, Germany.

2.2. Sol-Gel Entrapment Protocol – Method 1

This method was carried out as described in our previous work [17], using binary and tertiary mixtures of methoxysilane precursors, NaF as catalyst and [Omim]BF₄ as additive.

2.3. Sol-Gel Entrapment Protocol – Method 2

A *Candida antarctica* A lipase suspension (120 mg/mL) in TRIS/HCl 0.1 M, pH 8.0 buffer was stirred at room temperature for 30 min, centrifuged, and the supernatant was utilized for immobilization. 1 mL of this solution was mixed with 200 μ L [Omim]BF₄ and 200 μ L isopropyl alcohol in a 4 mL glass vial and magnetically stirred for 30 min. In a second 4 mL glass vial, the silane precursors (binary or tertiary mixture, total 6 mmoles), 0.4 mL water and 60 μ L 0.04M HCl were stirred for 30 min at

room temperature. Subsequently, the two solutions were mixed, 0.2 μ L ammonia (25% solution) has been added, and the whole mixture was stirred magnetically until the gelation occurred. The resulting gel was kept at room temperature for 24 h to complete polymerization. The bulk gel was washed successively with isopropyl alcohol (7 mL), distilled water (5 mL), isopropyl alcohol again (5 mL) and *n*-hexane (5 mL), filtered, dried at room temperature for 24 h, and in a vacuum oven at room temperature for another 24 h.

2.4. Acylation of secondary alcohols

Acylation were performed in 4 mL capacity glass vials, charged with a mixture of 2-hexanol, 2-heptanol or 2-octanol (0.5 mmole), vinyl acetate (1.5 mmole), internal standard (*n*-dodecane or *n*-decane, 15 μ L), reaction medium (*n*-hexane, 1 mL) and free (5 mg) or immobilized lipase (25 mg). The mixture was incubated using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40°C (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). The conversion and enantiomeric excess of the product were assayed by gas-chromatography, on a Varian 450 instrument (Varian Inc., USA) equipped with flame ionization detector, using a 30 m \times 0,25 mm Astec Chiraldex B-PM chiral column with 0.12 μ m film thickness (Sigma Aldrich, Supelco). The analysis conditions were: oven temperature from 100°C to 140°C with 10°C /min heating rate, injector temperature 240°C, detector temperature 280°C, carrier gas (hydrogen) flow 1.9 mL/min. The reactions were usually run for 24 h. Conversions have been calculated based on the internal standard method.

Transesterification activities were calculated at 24 h reaction time and expressed as the average 2-acetoxy-alcohol amount (in micromoles) synthesized per hour by 1 mg of native or immobilized enzyme. The control reaction without enzyme did not give any product in the same conditions. Relative total activities were calculated as the ratio of total enzymatic activity recovered following immobilization (activity of the immobilized enzyme multiplied by the amount of the immobilized preparete), divided by the total activity of lipase subjected to immobilization (activity of the native enzyme multiplied by the amount of native enzyme subjected to immobilization). The enantiomeric excess of the resulted ester product (ee_p) was determined from the GC peak areas of the enantiomers, and the enantiomeric ratio (E) values were calculated based on the conversion at 24 h C and ee_p , using the following equation [18]:

$$E = \frac{\ln[1 - C(1 + ee_p)]}{\ln[1 - C(1 - ee_p)]}$$

All reactions have been run in duplicate and sampling was also made in duplicate. As the differences between the data for the same assay were less than 2%, average values have been calculated and presented in the tables and figures.

3. Results and Discussion

Biocatalysts with high catalytic efficiency, selectivity and stability represent one of the important scientific challenges of the present days. CALA is not yet utilized in biocatalytic reactions at the same extent as CALB, but it is a very promising enzyme due to its thermal stability and selectivity toward substrates that are not transformed by other lipases, e.g. tertiary alcohols. In this study, CALA was immobilized by two sol-gel entrapment methods, using binary and ternary silane precursors systems, particularly octyl-trimethoxysilane/ tetramethoxysilane (OcTMOS:TMOS, at 1:1 molar ratio), phenyl-trimethoxysilane/ tetramethoxysilane (PhTMOS:TMOS, at 1:1 molar ratio), vinyl-trimethoxysilane/ tetramethoxysilane (VTMOS:TMOS, at 1:1 molar ratio), phenyl-trimethoxysilane/ methyl-trimethoxysilane/ tetramethoxysilane (PhTMOS:MeTMOS:TMOS, at 1.6:0.4:1 molar ratio) and phenyl-trimethoxysilane/ vinyl-trimethoxysilane/ tetramethoxysilane (PhTMOS:VTMOS:TMOS, at 1.6:0.4:1 molar ratio). The efficiency of different combinations of tetramethoxysilane and trimethoxysilanes holding phenyl, octyl, or vinyl nonhydrolyzable groups as starting materials for the sol-gel matrix was demonstrated in our previous studies for lipases from *Candida antarctica* B, *Pseudomonas fluorescens* and *Burkholderia cepacia* [17, 19-20]. The ionic liquid [Omim]BF₄ was employed as additive.

We studied the influence of immobilization method and the nature of the silane precursors on the catalytic activity and enantioselectivity of immobilized lipases. The acylation of aliphatic *sec*-alcohols (2-hexanol, 2-heptanol and 2-octanol) by vinyl acetate, in n-hexane at 40°C, was used as model reaction to evaluate the catalytic properties of the immobilized biocatalysts. The time courses of acylation of 2-hexanol, catalyzed by *Candida antarctica* A lipase, native and immobilized by *Method 1* and *Method 2* are presented in Fig. 1 and Fig. 2, respectively. The transesterification activities and enantiomeric excess values of the products are shown in Table 1 and 2, while the relative total activities are depicted in Fig. 3 and Fig. 4.

As seen from Fig. 1, for the enzyme immobilized by *Method 1* using as silane precursors PhTMOS:TMOS at 1:1 molar ratio, the conversion was almost total after 24h, in comparison with the native enzyme and the prepartate immobilized with VTMOS:TMOS (at the same molar ratio, 1:1), when the conversions at 24h reached only 53% and 55%, respectively. Obviously, the chemical structure of the nonhydrolyzable group of the silane precursor, retrieved unchanged in the sol-gel matrix, has an important influence on activity. The probable explanation is the different hydrophobicity of the phenyl compared to the vinyl group, an essential characteristic particularly in the case of reactions performed in non-polar media. Such an effect, increased activity of the lipase immobilized in sol-gels with higher phenyl group concentration, was previously observed for CALB, as well [17].

Method 2 of immobilization is based on the formation of a prepolymer sol, followed by gelation after switching the pH to basic with addition of ammonia. As shown in Table 1 and Fig. 2, this method resulted in less active prepartates compared to *Method 1* that used NaF as catalyst for the condensation and polycondensation reactions. The conversion after 24 h reached only 31% for the prepartate obtained with PhTMOS and TMOS (compared to > 99% when *Method 1* was applied with the same precursors) and even less (26%) for the prepartate based on a ternary silane mixture that included MeTMOS as the third precursor (PhTMOS:MeTMOS:TMOS, at 1.6:0.4:1 molar ratio).

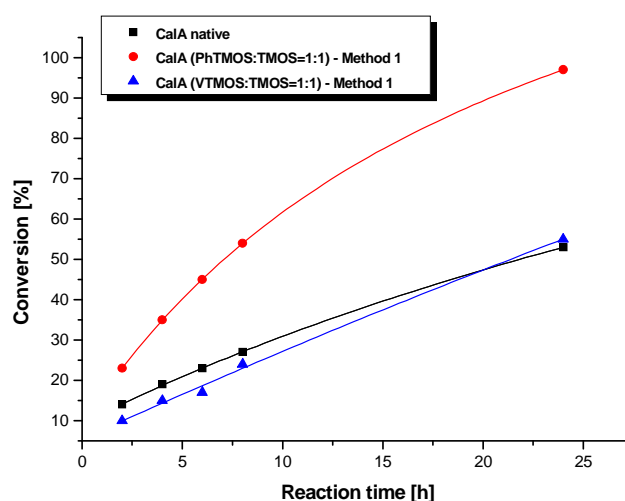


Figure 1. Time course of acylation of 2-hexanol with vinyl acetate in n-hexane at 40°C, catalyzed by native *Candida antarctica* A lipase (black) and immobilized by sol-gel entrapment - *Method 1*, with PhTMOS:TMOS at 1:1 molar ratio (red) and with VTMOS:TMOS at 1:1 molar ratio (blue)

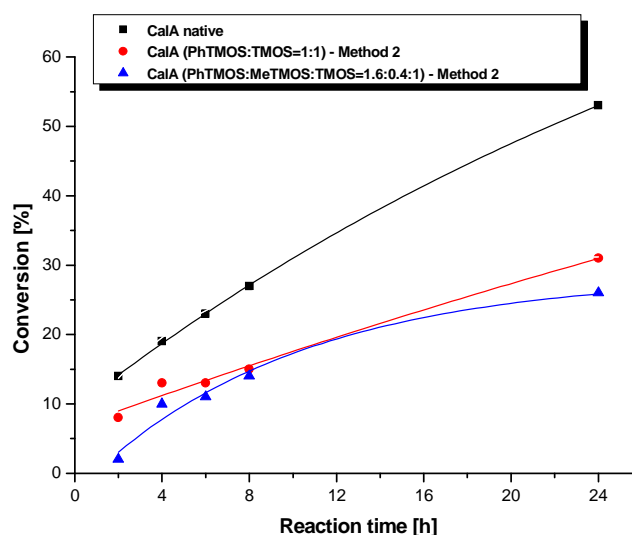


Figure 2. Time course of acylation of 2-hexanol with vinyl acetate in n-hexane at 40°C, catalyzed by native *Candida antarctica* A lipase (black) and immobilized by sol-gel entrapment - *Method 2*, with PhTMOS:TMOS at 1:1 molar ratio (red) and with PhTMOS:MeTMOS:TMOS at 1.6:0.4:1 molar ratio (blue)

TABLE 1. Influence of the immobilization method and silane precursor mixture composition on the activity of the sol-gel entrapped *Candida antarctica* A lipase in the acylation reaction of secondary aliphatic alcohols

Biocatalyst Silane precursors (molar ratio)	Activity ($\mu\text{mole}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$)					
	2-hexanol		2-heptanol		2-octanol	
	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
OcTMOS:TMOS (1:1)	0.825	0.710	0.720	0.726	0.808	0.865
PhTMOS:TMOS (1:1)	0.806	0.156	0.770	0.094	0.775	0.089
VTMOS:TMOS (1:1)	0.433	0.156	0.301	0.052	0.329	0.055
PhTMOS:MeTMOS:TMOS (1.6:0.4:1)	0.793	0.146	0.744	0.082	0.842	0.083
PhTMOS:VTMOS:TMOS (1.6:0.4:1)	0.705	0.102	0.720	0.042	0.834	0.042
Native CALA	0.854		0.348		0.490	

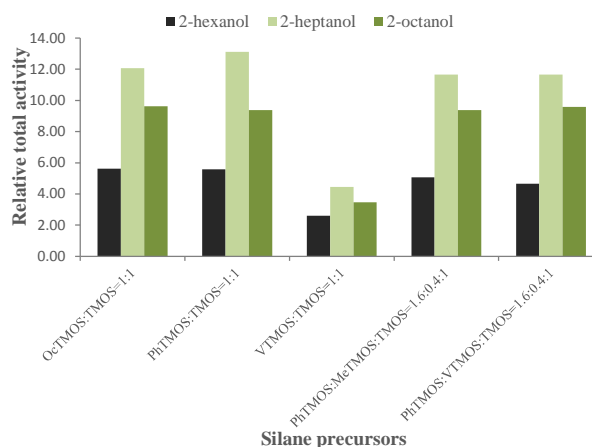
TABLE 2. Enhancement of *Candida antarctica* A lipase enantioselectivity, depending on the immobilization protocol. The enantiomeric excess ee_p values (%) were assayed for the kinetic resolution of racemic secondary alcohols

Biocatalyst Silane precursors (molar ratio)	Enantiomeric excess ee_p (%)					
	2-hexanol		2-heptanol		2-octanol	
	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
OcTMOS:TMOS (1:1)	2	-	-	1	-	1
PhTMOS:TMOS (1:1)	-	94	1	93	1	71
VTMOS:TMOS (1:1)	13	99	25	97	20	98
PhTMOS:MeTMOS:TMOS (1.6:0.4:1)	1	99	1	98	1	98
PhTMOS:VTMOS:TMOS (1.6:0.4:1)	1	99	1	97	-	98
Native CALA	8		2		2	

Table 1 demonstrates that most of the immobilized biocatalysts obtained by *Method 1* exhibited excellent activities for all tested substrates. The only exception was the prepare from VTMOS:TMOS at 1:1 molar ratio, with activities at about 50% or less compared to the other biocatalysts, depending on the substrate tested. These activities were remarkably high also compared with the native CALA, only 20% lower in the case of 2-hexanol substrate and up to 40% higher for 2-heptanol and 2-octanol. Such an increase of the activity (expressed as the amount of substrate transformed by 1 mg native or immobilized prepare) is not usual after the sol-gel entrapment, since the enzyme is distributed in a much larger matrix subsequent to the immobilization. The present results are better than previously reported for CALB in almost the same conditions [17], demonstrating the viability and efficiency of this immobilization method of CALA, even though these astonishingly high activities were associated with low enantioselectivity, as it will be shown.

In contrast, *Method 2* led typically to immobilized biocatalysts with much lower acylation activities in comparison to the native lipase. The exception was the prepare obtained with OcTMOS and TMOS precursors, that resulted in acylation activities for 2-heptanol and 2-octanol close to the values obtained with the prepares

obtained by *Method 1*, and considerably higher compared to the native CALA.

Figure 3. The immobilization method influence on the relative total activity of the immobilized *Candida antarctica* A lipase (*Method 1*), assayed in the acylation of secondary alcohols with vinyl acetate

The relative total activity was calculated in order to better emphasize the efficiency of the sol-gel immobilization, demonstrated by the much higher activity

values calculated for the whole amount of the immobilized prepate, compared to the total activity of the enzyme subjected to immobilization. A spectacular increase was observed for the biocatalysts immobilized with *Method 1*, up to 13 times higher compared to the native lipase for the best substrate, 2-heptanol, as shown in Fig. 3.

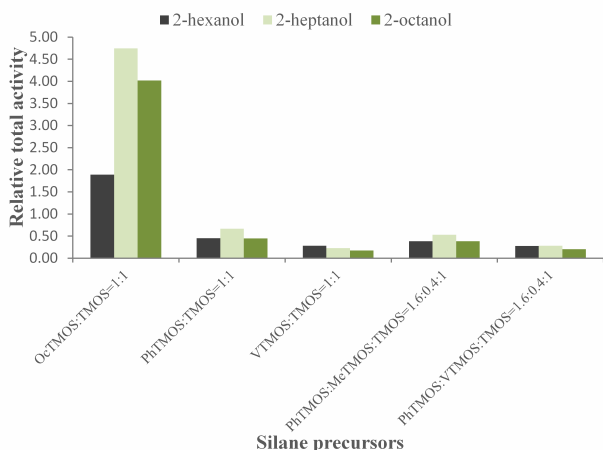


Figure 4. The immobilization method influence on the relative total activity of the immobilized *Candida antarctica* A lipase (*Method 2*), assayed in the acylation of secondary alcohols with vinyl acetate

For the biocatalysts obtained with *Method 2*, the relative total activity values were less than 1, meaning that part of activity was lost during the immobilization, or the access of the substrate to the entrapped enzyme is hindered. Another possible explanation of the activity loss observed after immobilization with *Method 2* could be some changes in the enzyme structure (a strong rigidification) that tend to decrease the catalytic activity, as found also by Rodrigues *et. al.* [13]. The prepate obtained with OcTMOS and TMOS was the exception, showing a relative total activity value exceeding 2. It can be presumed that the presence of the strongly hydrophobic octyl group in the matrix was beneficial for the lipase activity and influenced the structure of the obtained mesoporous material, facilitating the mass transfer to and from the active center.

High enantiomeric excess is another essential parameter for enzyme catalyzed kinetic resolutions and is particularly important for CALA, since this enzyme exhibited low enantioselectivity for the majority of previously tested substrates [21]. As seen from Table 2, the enantioselectivity values of the acylation reactions catalyzed by the immobilized prepatates obtained according to *Method 1* were very low, for all tested alcohols. The high tranacylation rates were practically the same for both enantiomers present in the racemate, like as in the case of the native CALA.

Opposite to *Method 1* of immobilization, *Method 2* gave biocatalysts (excepting the prepate obtained with OcTMOS and TMOS) that produced high enantiomeric excess values of the products and excellent enantiomeric ratios ($E > 110$, data not shown) for all tested substrates. When $E > 100$ it can consider that the chiral catalyst discriminates efficiently the enantiomers present in the racemate and the process is suitable for scaling-up. Based

only on these preliminary experimental data, it is impossible to explain such a high and surprising enantioselectivity. An excellent study performed by [21] demonstrated that a combinatorial library based engineering of CALA was successful to evidence the mutants with improved selectivity for the tranacylation of 1-phenylethanol, showing an E value increase from about 3 to more than 100 [21]. Our study revealed approximately the same increase of enantioselectivity only by the appropriate selection of the immobilization conditions.

These results demonstrate that immobilization of enzymes may be a very simple and powerful instrument to change the enzyme properties, and sol-gel entrapment can be such a choice.

4. Conclusions

The development of modern biotechnology includes the widespread application of biocatalysis, which increased the need for enzymes with improved operational stability and selectivity. In this context, immobilized biocatalysts became more and more efficient and enantioselectivity represents one of the most important characteristics for an immobilized enzyme to be used in large-scale processes, particularly in the pharmaceutical industry. The results of this study reveal that immobilization of enzymes may be a very simple and powerful instrument to change the enzyme properties, and sol-gel entrapment can be such a choice. By careful selection of the appropriate immobilization method and fine tuning the composition of the silane precursor mixture we demonstrated that the immobilization of CALA can be directed toward high tranesterification activity or high enantioselectivity. This lipase is not yet used in so many applications as CALB, but further research will certainly confirm its significant biocatalytic potential.

ACKNOWLEDGMENT

This work was performed through the Partnerships in priority areas - PN II program, developed with the support of MEN - UEFISCDI, project no. PN-II-PT-PCCA-2013-4-0734, and was partially supported by the strategic grant POSDRU/159/1.5/S/137070 (2014) of the Ministry of National Education, Romania, co-financed by the European Social Fund – Investing in People, within the Sectoral Operational Programme Human Resources Development 2007-2013.

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Received: 30 October 2015

Accepted: 27 November 2015