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Highly Efficient Synthesis of Glucose Fatty Acid Esters Catalyzed by High Performance Lipase Preparations

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Glucose fatty acid esters were synthesized using cross-linked enzyme aggregates (CLEAs), protein coated microcrystals (PCMCs) and cross-linked protein coated microcrystals (CLPCMCs) of *Candida antarctica* lipase B (CALB) as biocatalyst designs in single and mixed solvent systems. Up to 90% conversion and more than 99% regioselectivity were obtained using vinyl acetate as the acyl donor in a solvent system composed of 2-methyl-2-butanol (2M2B) and 30% (v/v) DMSO, with CALB CLEAs within 45 min. Similar results were obtained with CALB CLPCMCs as the biocatalyst under the same reaction conditions. This approach was then extended to the synthesis of glucose esters with higher acyl chain length. The synthetic strategy used in this work can potentially be extended for the fast and regioselective esterification/transesterification of other sugars as well.

Keywords: Lipase, Glucose-6-*O*-acetate, High activity biocatalyst designs, Transesterification, Regioselective synthesis.

INTRODUCTION

Fatty acid esters of sugars are widely used for industrial purposes as non-ionic surfactants [1,2]. These surfactants possess a range of properties such as foaming, wetting, emulsification, dispersion, *etc*. and hence find applications in food, cosmetic and pharmaceutical formulations [3-7]. Sugar esters are synthesized chemically by the regioselective esterification of one of the hydroxyl groups present in sugars using acidic and metal catalysts. This not only requires high temperature and high pressure, but also result in complex mixtures of multiple products [8]. Enzyme catalyzed synthesis of sugar esters offers a promising, less energy consuming alternative. Thus, recent years have seen a significant increase in the number of studies on the syntheses and applications of fatty acid esters prepared from mono- and disaccharides [9-14]. Enzymes, particularly lipases are known to catalyze regioselective and stereoselective acylation of alcohols in anhydrous organic solvents. In addition, enzymatic syntheses do not require high temperature and high pressure. Also, the purity of product(s) formed is higher. However, due to the limited solubility of sugars in most of commonly used anhydrous solvents, enzymatic esterification of sugars is often difficult to achieve. Sugars are reasonably soluble only in a few, hydrophilic organic solvents such as pyridine and dimethylformamide and most of the enzymes (especially the commonly used lipases) show poor activity in such solvents [15,16].

Over the years, various approaches have been employed to overcome this challenge. In one of the earliest approaches, Riva *et al.* [17] used proteases as biocatalysts in anhydrous DMF for catalyzing the transesterification reaction between sugars and trichloroethyl butyrate, resulting in regioselective acylation of the primary hydroxyl group of the sugar molecule. Subsequently, various examples of using proteases for sugar ester synthesis in highly hydrophilic solvents have been reported [18-20]. Another strategy for improving such syntheses is to use enol esters as the acylating agents. A number of hydroxy compounds including glycerol, various sugars and secondary alcohols have been acylated regio and/or stereoselectively using vinyl esters [21,22]. The alcohol produced in these reactions rapidly tautomerizes to acetaldehyde or acetone, which being volatile make the reaction irreversible. Thus, such transesteri-

fication reactions are faster, more selective and offer easier product isolation. This approach of using vinyl esters also finds application in the lipase catalyzed kinetic resolution of a large number of racemates [23].

However, unlike proteases, lipases get denatured in hydrophilic solvents. So, an alternate approach is used to employ lipases as biocatalysts for acylation of sugars. This involves the use of mixed solvents as reaction media. Generally, mixtures of *t*-alcohols (*t*-butanol or *t*-amyl alcohol) containing 5-30% (v/v) polar solvents like DMSO, DMF or pyridine are used [24-27]. The use of such mixed solvents is preferred as it enables a fine tuning of the solubility of the sugar and the enzyme activity. Lipases have proved to be efficient catalysts in such mixed solvent systems, resulting in high product yields as well as high regioselectivity.

Among all the commonly esterified sugars, glucose is expected to display a higher degree of regioselectivity since it contains only one primary hydroxyl group. The acylation of glucose catalyzed by *Candida antarctica* lipase B (CALB) is known to occur at the 1-°OH group followed by 2-°OH group at 3-*O*-position. Ljunger *et al.* [28] reported 99% conversion and 100% regioselectivity in the esterification reaction of glucose with octanoic acid after 24 h. Arcos *et al.* [8] used acetone as the medium for direct esterification of glucose with long chain fatty acids achieving 98% 6-*O*-lauryl glucose after 3 days. Cao *et al.* [29] carried out the regioselective solid phase synthesis of 6-*O*-palmitoylglucose with 86% yield being obtained after 48 h. Acylation of D-glucose with vinyl acetate using Novozyme 435 (immobilized CALB) has been studied by Park *et al*. [30] in a range of organic solvents and ionic liquids with varying degrees of regioselectivity being achieved depending on the reaction conditions. The use of ionic liquids as reaction medium for highly regioselective acylation reactions of glucose catalyzed by CALB has been reported elsewhere as well [31,32].

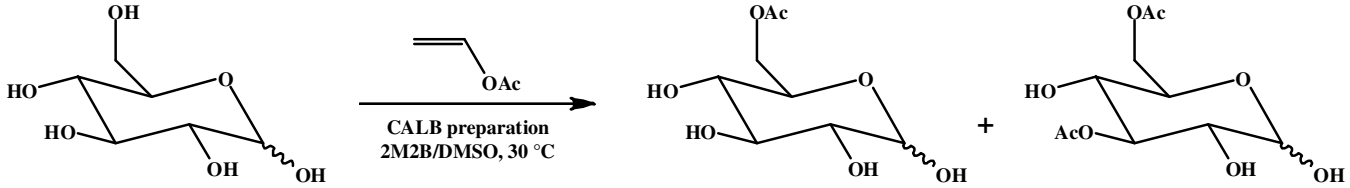
In present work, *Candida antarctica* lipase B (CAL-B) was chosen as its regioselectivity with respect to the hydroxyl groups of glucose has already been reported. A survey of the literature on sugar ester syntheses and their application as commercial surfactants led to two interesting observations. Firstly, there are quite a few reports in literature that describe the application of glucose esters as surfactants [33-35]. Secondly, although a variety of approaches have been used to improve the enzymatic route for production of sugar esters, high activity biocatalytic designs of enzymes (that are known to be effective catalysts for various reactions in low water media) have seldom been used for this purpose. In one such instance, the cross-linked enzyme aggregates (CLEAs) prepared from the *Thermomyces lanuginosus* lipase (TLL) were used for the synthesis of sucrose-6-acetate in a mixture of *t*-butanol and DMSO [27]. However, a comparative study about the use of different high activity biocatalysts for glucose ester synthesis has not been reported so far. Thus, we aimed at studying this relatively unexplored aspect of glucose ester synthesis that not only had the immense potential to enhance the reaction yields, but also decrease the reaction time. The biocatalyst designs chosen for this purpose were protein coated microcrystals (PCMCs), crosslinked enzyme aggregates (CLEAs) and crosslinked protein coated microcrystals (CLPCMCs). These biocatalyst formulations are prepared by precipitation of the enzyme using a suitable organic solvent and are found to give much faster rates as compared to the lyophilized enzymes [36-40]. **Scheme-I** shows the formation of 6-O-acetyl D-glucose and 3,6-diacetyl D-glucose in a reaction catalyzed by a highly active design of CALB. Under optimized conditions, these reactions could be carried out regioselectively and at rates that are far better than those already reported in literature.

EXPERIMENTAL

Lipozyme CALB (free form of *Candida antarctica* lipase B) was a kind gift from Novozymes, Bagsvaerd, Denmark. D-Glucose was purchased from Fischer Scientific, India. Vinyl acetate was obtained from Merck, Mumbai, India. Acetone, 2-methyl-2-butanaol (2M2B), DMSO (99.8% anhydrous grade), DMF, glutaraldehyde (25% v/v in water), N,O-*bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS) were obtained from Sigma, USA. All the solvents were dried over molecular sieves prior to use. Other chemicals were of analytical grade and used without further purification.

Synthesis of D-glucose esters in single and mixed organic solvents: D-glucose (90 mg, 0.5 mmol) and vinyl acetate (92 µL, 1 mmol) were added to 0.75 mL of the required solvent(s) taken in a vial sealed with Teflon tape. The reaction mixture was incubated at 55 ºC with orbital shaking at 300 rpm. The reaction was initiated by adding one of the biocatalyst formulations prepared from *Candida antarctica* lipase B (which was suspended in the same organic solvent) to the reaction vial so that the total volume was 1 mL. Orbital shaking was continued at 55 ºC with 300 rpm. Aliquots were withdrawn at different points of time and analyzed by GC.

Synthesis of D-glucose esters using solvent-premixing protocol: D-glucose (0.5 mmol) was added to 400 µL 2-methyl-2-butanol (2M2B) and required amount of DMSO (decided by the required final percentage composition of the reaction



D-glucose (mixture of anomers) | 6-O-Acetyl-D-glucose (mixture of anomers) | 3,6-O-Diacetyl-D-glucose (mixture of anomers)

Scheme-I: CALB catalyzed acetylation reaction of D-glucose with vinyl acetate

mixture). The mixture was incubated overnight at 55 ºC with 300 rpm. This resulted in a clear glucose solution. For initiation of reaction, the biocatalyst was suspended in 2M2B, containing 1 mmol of vinyl acetate and added to the above mixture so that the total volume was 1 mL. The reaction was then carried out at 55 ºC with orbital shaking at 300 rpm.

Preparation of cross-linked enzyme aggregates (CLEAs) of CALB: CLEAs of CALB were prepared by a reported method [41]. However, for the precipitation of enzyme, an aqueous enzyme solution was added to the excess organic solvent rather than adding organic solvent to the aqueous enzyme solution. This order of addition has been shown to result in enzyme preparations with lesser structural changes and hence greater catalytic activity [42]. A 1 mL of CALB solution was prepared (300 µL CALB + 700 µL 100 mM sodium phosphate buffer, pH 7.0). This enzyme solution was then added dropwise to 6 mL of dry chilled DME. The mixture was kept at 4 ºC, 150 rpm for 30 min for precipitation of proteins. This was followed by addition of required amount of glutaraldehyde (25 % v/v in water), so that the final concentration of glutaraldehyde was 150 mM. The mixture was then kept for 3 h at 4 ºC at 300 rpm. After this, the suspensions were centrifuged at 9000 g, 4 ºC for 10 min. The supernatant was decanted and the pellets of CLEA so obtained were washed three times with chilled DME (1 mL each time) for removal of unreacted glutaraldehyde. The DME was replaced by the desired organic solvent (which was to be used subsequently for the reaction) just prior to the setting up of the reaction.

Preparation of protein coated microcystals (PCMCs) and cross-linked protein-coated microcrystals (CLPCMCs) of CALB: The lipase PCMCs were prepared by the method described by Kreiner *et al.* [36]. CALB (300 µL) was diluted with 60 µL of 100 mM sodium phosphate buffer, pH 7.0. This solution was then mixed with 300 µL of saturated potassium sulphate solution and was cooled to 4 ºC. The solution was then added drop-wise to dry and chilled DME (4 mL) at 4 ºC, with constant orbital shaking at 150 rpm. The PCMCs were recovered after 30 min by centrifugation (9000 g, 5 min) at 4 ºC. These were then rinsed thrice with chilled dry DME. The DME was replaced by the desired organic solvent (which was to be used subsequently for the reaction) just prior to the setting up of the reaction.

For the preparation of CLPCMs, the PCMCs were suspended in DME (4 mL) by mild vortexing. To this, glutaraldehyde solution (25% v/v in water) was added so that the final glutaraldehyde concentration was 425 mM. The mixture was kept at 4 ºC for 1 h with constant shaking at 300 rpm. The CLPCMs were recovered by centrifugation at 9000 g, 5 min at 4 ºC and were washed with ice chilled dry DME thrice to ensure that unreacted glutaraldehyde was removed completely from the enzyme preparation.

Derivatization of glucose and its esters for GC analysis: Aliquots of 20 µL were withdrawn at various points of time, vacuum dried to remove the solvents and then silylated for GC analysis. For derivatization, 100 µL pyridine (containing 1 mg tetradecane as internal standard) and 200 µL BSTFA (containing 1% TMCS) were added to each vacuum dried aliquot. The resulting mixture was then incubated at 70 ºC for 20 min. The sample was then cooled to room temperature and 1 µL from this was injected into GC.

GC analysis: Trimethylsilyl (TMS) derivatives of glucose and its esters were analyzed on Agilent Technologies 6890 N Network, USA, equipped with a flame ionization detector. A non-polar, fused silica capillary column, Equity™-5 (5% diphenyl-95% dimethyl polysiloxane) (30 mm × 0.32 mm × 0.25 µm) was used for analysis. The temperature program used was according to the described earlier [43]. For determination of calibration curves, the derivatized samples of pure glucose, 6-*O*-acetyl-D-glucose and 3,6-*O*-diacetyl-D-glucose were injected.

Characterization: The position of acetylation of glucose was confirmed by NMR spectroscopy. ^{1}H & ^{13}C NMR spectra of the monoester and diester were recorded on Bruker-S-300 spectrometer and matched with those reported in literature [44].

RESULTS AND DISCUSSION

Regioselectivity in sugar fatty acid ester synthesis: The transesterification reaction of D-glucose and vinyl acetate was studied in different reaction media. Polyhydroxy compounds such as glucose are appreciably soluble only in polar media [17]. However, such solvents with log P value below 2 are not considered suitable for enzyme catalysis [45]. Table-1 lists some organic solvents (along with their log P values) which are commonly used as reaction media for sugar ester synthesis. The rates of CALB catalyzed glucose ester synthesis in highly hydrophobic solvents such as hexane and toluene have been reported to be very low due to the extremely limited solubility of glucose in these solvents [46]. In present work, screening of the solvents, however solvents with low hydrophobicity (*i.e.* log P > 2) were considered.

TABLE-1
ORGANIC SOLVENTS WITH DIFFERENT HYDROPHOBICITY THAT ARE COMMONLY USED AS REACTION MEDIA FOR SUGAR FATTY ACID ESTER SYNTHESIS

Organic solvent	Solvent hydrophobicity (log P)
DMSO	-1.35
DMF	-1.01
Acetonitrile	-0.33
1,4-Dioxane	-0.27
Acetone	-0.24
t-Butanol	0.37
THF	0.46
t-pentanol (or *t*-amyl alcohol or 2-methyl-2-butanol)	0.89

To begin with acetone (log P = -0.24) was chosen as the solvent to evaluate the performance of different biocatalyst designs of CALB. Acetone has a low boiling point which facilitates easy work up and also considered to be a safe solvent for the manufacture of food and pharmaceuticals [47]. Reactions catalyzed by three biocatalyst designs were carried out in acetone as the solvent for 15 h. The results obtained are summarized in Table-2. It was found that while in terms of overall conversion of D-glucose, CLEA and CLPCMC (which gave 87% and 95% conversions, respectively) were found to be better

TABLE-2
ACETYLATION REACTION OF GLUCOSE CATALYZED BY DIFFERENT BIOCATALYST DESIGNS OF CALB IN ACETONE AS THE REACTION MEDIUM

Biocatalyst design	Time (h)	Conversion[a] (%)	6-*O*-Acetyl-D-glucose[b] (%)	3,6-*O*-Diacetyl-D-glucose[c] (%)	Monoacetylation[d] (%)
PCMC	15	21	20	1	93
	24	36	33	3	92
CLPCMC	15	95	75	20	79
	24	100	70	30	70
CLEA	15	87	72	15	83
	24	91	64	27	70

[a] Total conversion (monoacetate + diacetate) obtained after a period of 15 h by GC analysis. [b] Conversion (%) to the monoacetate. [c] Conversion (%) to the diacetate. [d] Ratio of monoacetate/diacetate. The experiments were done in triplicate and the percentage error in each case was ± 5%.

than PCMC, yet the reactions catalyzed by these biocatalysts were less regioselective, with the respective monoacylation being 83% and 79%. Also, if the reaction time was extended from 15 h to 24 h, then subsequent conversion of monoacetate to diacetate was observed. With PCMC as the biocatalyst, although the overall conversion observed after 15 h was only 21%, yet the regioselectivity was comparatively better with 93% monoacylation being attained.

In acetone and other solvents with similar polarity, glucose has limited solubility. The initial acetylation (of whatever glucose was in the dissolved form) yielded 6-*O*-acetylated compound, which was more soluble in acetone than the parent sugar. Thus, further acetylation preferentially occurred on this monoacetylated glucose, resulting in the formation of 3,6-*O*-diacetyl-D-glucose. This accounts for the low regioselectivity observed in general for such solvents [8,30].

Subsequently, two more solvents were chosen for evaluating the performance of these biocatalyst designs: one less polar than acetone and the other more polar than acetone. These were *t*-butanol (log P = 0.37) and DMF (log P = -1.01). In *t*-butanol (like in acetone), CLEA and CLPCMC of CAL-B resulted in > 90% acetylation of glucose after 15 h. However, the regioselctivity shown by these biocatalysts was poor in these solvents as well. In DMF (high polar solvent), the enzyme lost activity and very low conversions were achieved, but the regioselectivity was close to 100% as negligible amount of the diacetate was formed. Fig. 1 shows the comparative performance of the three biocatalyst designs in these solvents. Clearly, none of the solvents used so far helped in the attainment of high reaction rates coupled with high regioselectivity. Table-3 summarizes the initial rates of glucose conversion obtained in these solvents. It can be seen that in this case increasing polarity of the reaction medium led to decreasing initial rates for the conversion of glucose by all three biocatalyst designs. Also, the performance of CLPCMC was better than that of PCMC and CLEA in acetone and *t*-butanol. However, in DMF as the reaction medium, CLEA gave the best results.

Degn *et al.* [48] reported reaction rates of 1.6 ± 0.4 (μmol min^{-1} g^{-1}) for the direct esterification of glucose and acetic acid in *t*-butanol using Novozyme 435 as the biocatalyst. In the present case, using the same reaction medium gave initial rates of 681.8 and 631.3 μmol min^{-1} g^{-1} with CLPCMC and CLEA, respectively. Degn & Zimmermann [46] reported a maximum

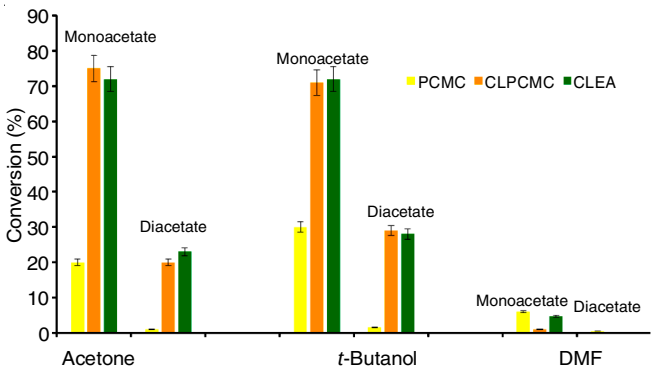


Fig. 1. Effect of solvent hydrophobicity on the CALB catalyzed acetylation of glucose. Three solvents: acetone (log P = -0.24), *t*-butanol (log P = 0.37) and DMF (log P = -1.01) were used as the reaction media. Monoacetate refers to 6-*O*-acyl-D-glucose and diaceatate refers to 3, 6-*O*-diacyl-D-glucose. Conversion (%) was measured by GC analysis after a period of 15 h. Experiments were done in triplicate and error in each set of readings was ± 5%

TABLE-3
INITIAL RATES FOR THE TRANSESTERIFICATION OF GLUCOSE AND VINYL ACETATE IN SOLVENTS OF DIFFERENT POLARITY[a]

Solvent (log P)	Biocatalyst design	Initial rate (μmol min^{-1} g^{-1})[b]
t-Butanol (0.37)	PCMC	75.7
	CLPCMC	681.8
	CLEA	631.3
Acetone (-0.24)	PCMC	45.4
	CLPCMC	366.1
	CLEA	353.5
DMF (-1.01)	PCMC	12.6
	CLPCMC	7.5
	CLEA	35.1

[a] Three solvents of varying polarity (as measured by the log P values) were used. In each solvent, three biocatalysts: PCMC, CLPCMC and CLEA were used. [b] Initial rates were calculated from aliquots taken within first 30 min of the reaction. % Conversions during this time increased linearly. The experiments were done in triplicate and the percentage error in each case was ± 5%.

enzyme activity of 20.2 μmol min^{-1} g^{-1} for the esterification of glucose with myristic acid. Elsewhere using the same biocatalyst in presence of excess glucose, with 2-methyl-2-butanol as the reaction solvent and lauric acid as the acyl donor, initial rates of 8.6 μmol min^{-1} g^{-1} have been reported [49].

Mixed-solvent approach for glucose ester synthesis: Of the many approaches used to improve sugar solubility while maintaining the enzyme stability, the use of mixed-solvents is a relatively simple yet effective one. Recently, CALB catalyzed synthesis of esters of glucose and fatty acids such as palmitic acid, lauric acid and hexanoic acid in solvent mixtures comprising of DMSO and 2-methyl-2-butanol has been reported [50]. The researchers have also evaluated the functionality of these esters as emulsifiers. As mentioned earlier, one of the advantages of using such mixed-solvent systems is that polarity of the reaction medium can be gradually changed by stepwise increase in percentage of the polar co-solvent (generally DMSO, DMF or pyridine) without bringing about drastic changes in environment of the enzyme. This is generally found to favour high regioselectivity. Degn & Zimmnermann [46] have successfully used this approach for synthesis of myristyl glucose. Disaccharide esters have also been prepared by various researchers by employing mixed solvents [25,26,51].

Thus, in present case, it was decided to investigate the effect of using such mixed-solvents for the reaction of glucose and vinyl acetate. Binary mixtures of 2M2B and DMSO were chosen as the reaction media. The two biocatalyst designs: CLEA and CLPCMC, which had performed better (in terms of overall conversion) earlier were used here. Table-4 shows the performance of CLEAs prepared from CALB in the reaction media containing varying amounts of DMSO. As seen, increasing the DMSO content led to an increase in the % monoacylation (ratio of monoacetate/diacetate). This enhancement in the regioselectivity of the reaction can be attributed to the greater solubility of glucose in presence of high DMSO concentration [47]. In terms of the overall conversion (monoacetate + diacetate), maximum production of glucose esters took place at 30% (v/v) DMSO. However, beyond this concentration of DMSO, the overall conversions decreased in spite of high glucose solubility. This is due to the denaturing effect of high concentration of polar organic solvents on activity and stability of lipases [46,51]. An einitial reaction rate of 3636 μmol min^{-1} g^{-1} was achieved at 30% DMSO concentration.

A similar performance was shown by the CLPCMCs prepared from CALB in solvent mixtures of 2M2B and DMSO (Table-5). With this biocatalyst design as well, the 30% DMSO concentration proved to be the optimum both in terms of regioselectivity as well as overall conversion. An initial rate of 3333 μmol min^{-1} g^{-1} was attained at this solvent composition.

Preparation of high activity biocatalyst designs of CALB: The limited activity of 'straight from the vendor' enzyme preparations in low water media has always been a matter of concern while utilizing enzymes in synthetic organic chemistry. High activity biocatalyst designs have helped in overcoming this limitation to a large extent. Three of these biocatalyst designs have been used in this work. These are protein coated microcrystals (PCMCs), crosslinked protein coated microcrystals (CLPCMCs) and crosslinked enzyme aggregates (CLEAs). An initial screening of these three biocatalysts in single solvents showed CLEA to be a better biocatalyst design (Fig. 2); while in mixed solvent system also the performance of CLEA was

TABLE-4
EFFECT OF INCREASING CONCENTRATION OF POLAR CO-SOLVENT ON THE SYNTHESIS OF 6-*O*-ACETYL AND 3,6-*O*-DIACETYL- D-GLUCOSE CATALYZED BY CLEAs OF *Candida antarctica* LIPASE B

DMSO (co-solvent) concentration[a] (%)	Initial rate (μmol min^{-1} g^{-1})[b]	Conversion[c] (%)	6-*O*-Acetyl-D-glucose (%)[d]	3,6-*O*-Diacetyl-D-glucose (%)[e]	Monoacylation (%)[f]
0	151	6	4.2	1.8	70
5	454	15	11.5	3.5	77
10	909	22	18	4	81
20	2424	30	27	3	90
30	3636	43	43	0	100
40	960	28	28	0	100

[a] Solvent mixtures were prepared in v/v proportions. [b] Initial rates were calculated from aliquots taken within first 30 min of the reaction. % Conversions during this time increased linearly. [c] Total conversion (monoacetate + diacetate) obtained by GC analysis obtained after a period of 1 h. [d] Conversion (%) to the monoacetate. [e] Conversion (%) to the diacetate. [f] Ratio of monoacetate/diacetate. The experiments were done in triplicate and the percentage error in each case was ± 5%.

TABLE-5
EFFECT OF INCREASING CONCENTRATION OF POLAR CO-SOLVENT ON THE SYNTHESIS OF 6-*O*-ACETYL AND 3, 6-*O*-DIACETYL-D-GLUCOSE CATALYZED BY CLPCMCs OF *Candida antarctica* LIPASE B

Co-solvent concentration[a] (%)	Initial rate (μmol min^{-1} g^{-1})[b]	Conversion[c] (%)	6-*O*-Acetyl-D-glucose (%)[d]	3,6-*O*-Diacetyl-D-glucose (%)[e]	Monoacylation (%)[f]
0	126	4.8	3.6	1.2	75
5	404	14	11	3	78
10	859	19	16	3	84
20	2222	28	26	2	93
30	3333	39	39	0	100
40	909	26	26	0	100

[a] Solvent mixtures were prepared in v/v proportions. [b] Initial rates were calculated from aliquots taken within first 30 min of the reaction. % Conversions during this time increased linearly. [c] Total conversion (monoacetate+ diacetate) obtained by GC analysis obtained after a period of 1 h. [d] Conversion (%) to the monoacetate. [e] Conversion (%) to the diacetate. [f] Ratio of monoacetate/diacetate. The experiments were done in triplicate and the percentage error in each case was ± 5%.

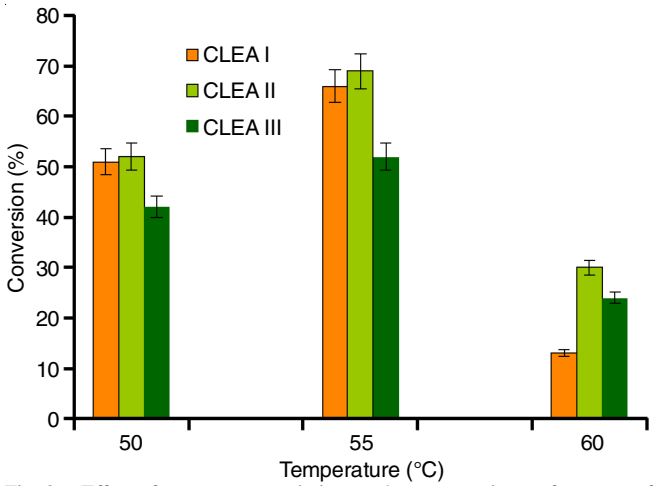


Fig. 2. Effect of temperature variation on the comparative performance of CLEAs of *Candida antarctica* lipase B. CLEAs I, II and III were prepared by using 50mM, 150mM and 250 mM glutaraldehyde (as cross-linking agent) respectively. Transesterification reaction of glucose with vinyl acetate was carried out in a solvent mixture composed of 2M2B and 30% DMSO at different temperatures: 50, 55 and 60 °C. At each reaction temperature, all three CLEA designs were used. Reaction time was 15 h. Experiments were done in triplicate and the error in each set of readings was ± 5%

better than CLPCMC (Tables 4 and 5). Designing of CLEAs essentially involves two steps: precipitating the enzyme by using a water-soluble organic solvent, followed by crosslinking the precipitated enzyme using a suitable crosslinking agent [42]. Although, a number of water-soluble organic solvents are available for enzyme precipitation, DME is known to be the best precipitant for CALB as with this 100% activity is retained in the precipitate [37]. Thus, DME was the organic solvent chosen by us for precipitation of CALB. The next step in CLEA preparation involves the addition of a cross-linker, one of the most commonly used crosslinking agents being glutaraldehyde. Optimization of the amount of glutaraldehyde added is crucial in this step. In case of CALB, higher glutaraldehyde concentrations (upto 150 mM) are reported to lead to hyperactivation [41]. The transesterification reaction of D-glucose and vinyl acetate was carried out with CALB CLEAs prepared using three glutaraldehyde concentrations: 50, 150 and 250 mM. These have been designated as CLEA I, CLEA II and CLEA III, respectively.

Choice of reaction temperature: The transesterification reaction of glucose catalyzed by the CLEAs of CALB in 2M2B containing 30% DMSO was carried out at 50, 55 and 60 ºC. Fig. 2 shows the comparative performance of the three CLEAs at these temperatures. As the temperature was increased from 50 to 55 ºC, the catalytic activity of all the CLEAs increased, which is reflected in the increased conversion values. At 55 ºC, of all the CLEAs, CLEA II gave the maximum conversion. However, as the temperature was increased to 60 ºC, the performance of all the CLEAs decreased drastically. Thus, for further experiments, CLEA II was retained as the biocatalyst design with the reaction temperature being 55 ºC.

Solvent-premixing protocol: Once the biocatalyst design and reaction temperature were optimized, a 'solvent-premixing protocol' was used for dissolving glucose in the reaction medium. Ballesteros *et al*. [26] have used a similar solvent-mixing method for the synthesis of sucrose esters, at high substrate loadings. We decided to employ a 'solvent pre-mixing' protocol in conjunction with the use of high activity biocatalyst designs of CALB for esterification of glucose. It was found that 'premixing' the glucose in 2M2B and DMSO and keeping the mixture at 55 ºC, 300 rpm overnight resulted in complete dissolution of glucose. Addition of the biocatalyst (suspended in minimum volume of 2M2B) along with the acyl donor to this clear solution resulted in an extremely fast con-version of glucose to glucose monoacetate.

Fig. 3 shows the time course for the regioselective acylation of glucose catalyzed by CLEA of CALB in 2M2B with different concentrations of DMSO. An increase in the amount of DMSO from 10 to 30% (v/v) led to better rates of formation of the glucose esters. Also, the maximum conversion of glucose was obtained at this DMSO concentration. Glucose was exclusively converted to its monoacetate by following this protocol. It was observed that > 90% of glucose was converted to the product within a period of just 90 min. In addition, > 99% of this product was composed of the monoacetate. Extending the reaction for longer duration beyond this time led to gradual conversion of the monoacetate to the diacetate, without much change in the overall conversion.

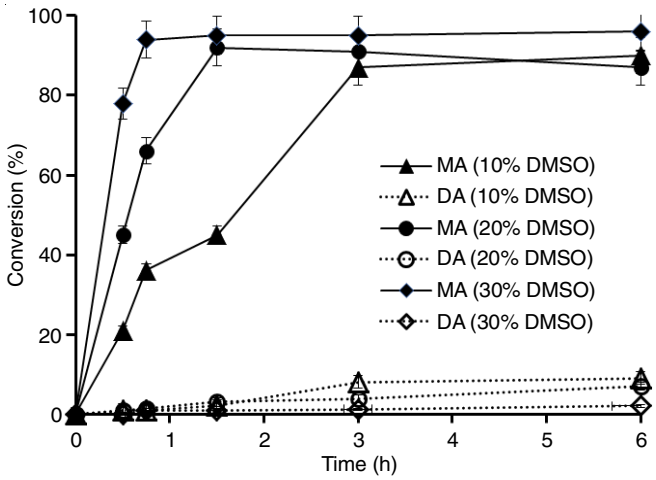


Fig. 3. Time course for the CALB CLEA catalyzed regioselective acetylation of D-glucose carried out using 'solvent-premixing' protocol. Reactions were carried out in 2M2B containing three different DMSO concentrations: 10%, 20% and 30% (v/v), using CLEA III (prepared using 150 mM glutaraldehyde) as the biocatalyst design. Experiments were carried out in triplicate and error within each set of readings was ± 5%. Reaction aliquots taken over different points of time were analyzed by GC. MA refers to monoacetate and DA refers to diacetate

Next step was to use CLPCMC as a biocatalyst and determine its performance under these conditions. As shown in Fig. 4, with this biocatalyst design as well, the acylation reaction proceeded fastest and most regioselectively in the reaction medium consisting of 2M2B and 30% (v/v) DMSO. Table-6 summarizes the initial rates for glucose ester synthesis using solvent-premixing protocol. In the reaction medium containing 2M2B and 30% DMSO, initial rates of 7886.9 and 7447.5 μmol min^{-1} g^{-1} were obtained using CLEA and CLPCMC,

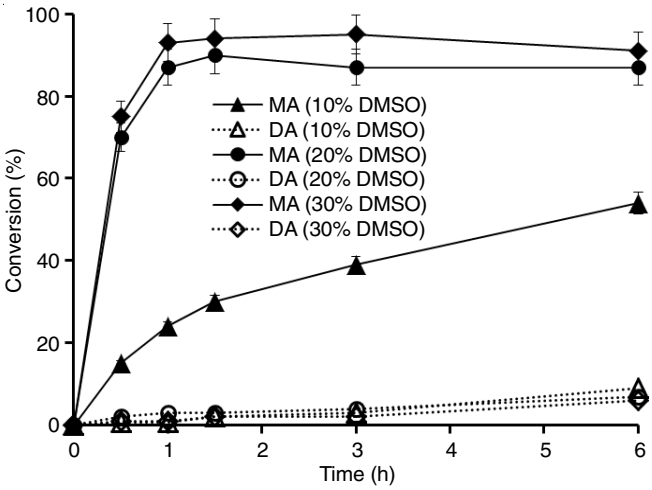


Fig. 4. Time course for the CALB CLPCMC catalyzed regioselective acetylation of D-glucose carried out using 'solvent-premixing' protocol. Reactions were carried out in 2M2B containing three different DMSO concentrations: 10 %, 20% and 30% (v/v) using CLPCMC as the biocatalyst. Experiments were carried out in triplicate and error within each set of readings was ± 5%. Reaction aliquots taken over different points of time were analyzed by GC. MA refers to monoacetate and DA refers to diacetate

TABLE-6
INITIAL RATES FOR GLUCOSE ESTER SYNTHESIS CARRIED OUT USING SOLVENT-PREMIXING PROTOCOL

Solvent composition	Biocatalyst design	Initial rate ($\mu mol\ min^{-1}\ g^{-1}$)[a]
2M2B + 10%DMSO	CLEA	2424.1
	CLPCMC	2100.3
2M2B + 20%DMSO	CLEA	4500.0
	CLPCMC	4400.1
2M2B + 30%DMSO	CLEA	7886.9
	CLPCMC	7447.5

[a] Initial rates were calculated by the total conversion of glucose within the linear range. The experiments were done in duplicate and the percentage error in each case was ± 5%.

respectively. Clearly, the synergistic effect of the reaction conditions employed as well as the use of high activity biocatalyst designs enabled the reaction to proceed at rates which are way ahead of those already reported in literature.

Acylation reaction of glucose with different acyl donors: In order to further explore the scope and generality of this reaction, the acylation reaction of glucose was carried out with different acyl donors under the previously optimized conditions. Isopropenyl acetate is an efficient and cost-effective acylating agent for hydroxyl and amino groups [52]. It is commonly used as an acetyl source as acetone released as a byproduct is a volatile and reusable solvent which shifts the equilibrium to the right. Thus, the acylation of glucose was performed with isopropenyl acetate using the CLEA of CALB as the biocatalyst in a mixture of 2M2B and 30% DMSO following the solvent premixing protocol. Also, we screened vinyl esters of propionic acid, butanoic acid and lauric acid as acyl donors for glucose (Table-7). All the acyl donors resulted in > 99% regioselectivity. The overall conversion obtained after 45 min with isopropenyl acetate was slightly lesser than that obtained with vinyl acetate. With other acyl donors, under our reaction conditions,

TABLE-7
EFFECT OF VARYING THE ACYL DONOR ON THE CALB CLEA CATALYZED ACYLATION OF GLUCOSE[a]

Acyl donor	6-*O*-Acyl-D-glucose (%)[b]	3,6-*O*-Diacyl-D-glucose (%)[c]	Mono-acylation (%)
Vinyl acetate	60	0.2	> 99
Isopropenyl acetate	54	0.6	> 99
Vinyl propionate	66	0.5	> 99
Vinyl butyrate	72	1.0	> 99
Vinyl laurate	81	0.9	> 99

[a] Reactions were carried out in 2M2B containing 30% DMSO (v/v) as the reaction medium for a period of 45 min, using solvent premixing protocol. [b] Conversion (%) to the monoacetate. [c] Conversion (%) to the diacetate. The experiments were done in duplicate and the percentage error in each case was ± 5%.

the initial rates of glucose esterification reaction did not change with increase in the donor chain length. A similar trend has also been observed by Deng *et al*. [48] who esterified glucose using immobilized CALB in *t*-butanol. Ren & Lamsal [50] also reported that the initial rate of the reaction of glucose is independent of the alkyl chain length. Recently, esterification of D-glucose and fatty acyl amino acids catalyzed by free CALB was reported [53]. The authors observed that increasing the chain length of the hydrophobic part of the ester molecule lowered its critical micelle concentration (CMC) and surface tension (γ_{cmc}). Glucose laurate synthesis accompanied by high productivity has also been carried by the lipases from *Aspergillus niger* and *Aspergillus oryzae* apart from the *Candida antarctica* lipase B [54]. Under optimized reaction conditions, the authors obtained a maximum of about 40% conversion within 5 h in the reaction catalyzed by Novozyme 435. In the present work, under present optimized conditions, 81% conversion to glucose laurate along with > 99% regioselectivity was achieved within 45 min of the reaction.

Conclusion

The use of cross-linked enzyme aggregates (CLEAs) as a biocatalyst design for the synthesis of a few sucrose esters has been reported by some researchers. However, to the best of our knowledge, there has been no previous comparative study about the use of high activity biocatalyst designs for such reactions. Also, as mentioned earlier, the esterification/transesterification of glucose has not been performed so far with these biocatalyst designs. The results described in this work indicate that by employing high activity enzyme preparations under the optimized reaction conditions, it is possible to achieve a high percentage of the glucose esters, accompanied by excellent regioselectivity within a very short period of time. Although, the present work has been limited to glucose as acyl acceptor, yet the synthetic strategy developed herein holds a lot of promise which can be applied for production of esters of other monosaccharides and disaccharides.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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