Asian Journal of Chemistry; Vol. 25, No. 6 (2013), 3014-3018

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13492

Lipase-Catalyzed Amino Sugar Derivative in Tri-solvent Mixture

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(*Received*: 21 January 2012; *Accepted*: 7 December 2012) AJC-12509

In this work, a new type of amino sugar derivative tagged as amino sugar similar to N-acetyl-glucosamine, commonly used in medical field as medicine to treat osteoarthritis has been synthesized. This amino sugar derivative produced from glucose and propylamine with the aid of immobilized *Candida antarctica* (Novozyme 435) lipase, as catalyst. Mixtures of solvent influence the high solubility of sugar and thus increasing the yield. Optimization studies such as time, molar ratio, temperature and enzyme quantity conducted for optimization. Optimized condition yields 73.37 % amino sugar was obtained upon 2 h of reaction, 1:5 molar ratio of substrates, 40 ºC temperature and 17 % enzyme mass.

Key Words: Lipase, Glucose, Amino sugar, Ionic liquid, Enzymatic.

INTRODUCTION

Sugar esters are non-ionic surfactants which have diverse uses commercially. It is most prominently used in food industries around the globe as flavouring agent. Other sectors such as medical, cosmetics, oral-care products and detergents employed sugar esters as surface active agent[1,2]. Amino sugars are new subject in esters synthesis whereby substrate with an amino group as amination reagent is reacted with glucose.

Commonly amino sugars were synthesized using various forms of amino acids. For example in the food and pharmaceutical industries, L-aspartic acid is used to form esters (aspartame) which used as artificial sweetener[3]. In pharmaceutical industries, amino sugar derivatives could function as antibiotics, active nucleoside and as transporter of biological active agents.

Amino sugar derivative (N-acetyl-glucosamine) is synthesized naturally in our body with the aid of chondrocytes which form glucosamine precursor. Most amino sugars are synthesized by employing sugars and amino acids as reactants. However, apart from trying to synthesize amino sugars, it is also obtained from natural sources. This process certainly does not support on preserving nature's resources as most often amino sugars such as D-glucosamine is obtained by hydrolysis or fermentation on crustacean exoskeletons[4].

In present work, we tried to work beyond the dependence on amino acids or naturally occurring amino sugars which are mostly obtained from mammals, by using base (propylamine) in this research. This reaction was conducted in tri-solvent mixture consisting of ionic liquid [BMIM][PF_6], dimethyl sulfoxide (DMSO) and *tert*-butanol.

A most prominent problem in glycotechnology is the difficulty of dissolving sugars in organic solvents. Many research aim in finding the correct remedy to achieve bi-functional target to dissolve sugars and achieve high percentage of esters yield. DMSO and DMF are favourite solvents in glycotechnology due to their low log P value (hydrophilic) giving high solubility rate[5].

Gulati *et al.*[6] on the other hand employed only *tert*-butanol causing low reaction rate due to sugars low solubility factor, which corresponds to high log P value (hydrophobic) of the solvent media. Later Ferrer and his team synthesized sugar ester in solvent mixtures consisting DMSO and *tert*-butanol promising bi-functional attributes[7]. High sugar solubility due to low log P value of DMSO and high reaction rate caused by high log P of *tert*-butanol value gives good conversion rates.

The usage of ionic liquid, DMSO and *tert*-butanol in this research was with the aim of achieving high solubility of sugars[8,5,9]. Ionic liquid's motive in this research is to give positive impact to esterification reactions in order to its ability to dissolve inorganic substances and most importantly environmental friendly compared to organic solvents which have high vapour pressure[10,11].

EXPERIMENTAL

The immobilized enzymes Novozyme 435, Lipozyme RM IM and Lipozyme TM IM were purchased from Novozymes, Denmark. Crude lipase from *Candida rugosa* and D-(+)-glucose were purchased from Sigma-Aldrich, United States. Propylamine was purchased from Fluka, Switzerland. Ionic liquid, 1-butyl-3-methyl-imidazolium hexafluorophosphate was purchased from Merck, Germany. All other chemicals and solvents used in this research were of analytical grade.

Screening of enzyme for lipase-catalysed reaction: Crude lipase activity was screened for lipase-catalysed amino sugar derivative reaction together with several other immobilized lipases (Novozyme 435, Lipozyme RM IM, Lipozyme TL IM).

Lipase-catalysed synthesis of amino sugar derivative: In a capped vial, D-(+)-glucose (0.5 mmol) was added to mixture of solvents (12.5 % ionic liquid: 37.5 % DMSO). The reactant was then dissolved at 60 ºC water bath. Addition of 50 % *tert*-butanol was performed prior to the mixing of the visible biphase. 3 Å molecular sieves, 17 % of enzyme, propylamine (0.5 mmol) and a magnetic bar were added into the same vial before reaction proceeded in oil bath at 300 rpm at specified period of reaction and temperature.

Determination of amino sugar derivative by DNSA method[12]: Reaction product with a volume of 0.2 mL, was pipette into a vial together with 0.6 mL of DNSA. The resulting solution was then left in 60 ºC water bath. 25 mL of distilled water was added to the sample and control vials followed by UV analysis on their absorbance at 540 nm. The moles of glucose reacted in this reaction were determined by using the absorbance value of control, samples and standard curve calibrated using standard glucose solutions. The amount of amino sugar derivative formed is calculated from the equation 1.

$$\text{Percentage of amino sugar conversion} = \frac{\text{Concentration of control} - \text{Concentration of sample}}{\text{Concentration of control}} \quad (1)$$

Time parameter study: Influence of reaction duration in amino sugar derivative synthesis was investigated in this research. This was performed by varying the reaction time at 30, 60, 120 and 240 min followed by conversion calculation.

Molar ratio study: Effect of different amount of base (substrate) in this reaction ranging from 0.5-3.5 mmol was evaluated.

Reaction temperature study: Temperature effect on this reaction was evaluated. Chosen temperatures for this amino sugar derivative synthesis reaction were 30, 40 and 50 ºC.

Quantity of enzyme study: Different quantity of enzyme for optimal yield of amino sugar product was investigated. The amount of enzyme tested were 1, 2 and 3 mg.

Mass spectroscopy analysis: Ion fragments of the product were identified using gas chromatography mass spectrometer (Shidmadzu) model QP5050A.

RESULTS AND DISCUSSION

Solvent screening: In understanding on the importance of solvent polarity for dissolving sugars (polar), various types of solvents was screened. Table-1 shows the solvents screened for dissolving glucose prior to reacting with propylamine.

TABLE-1
SOLVENT SCREENING FOR SUGAR DISSOLVING

Solvent	Nature	log P	Solubility
Dimethyl sulfoxide (DMSO)	Polar	-1.378	Excellent
Acetone	Polar	-0.208	Fair
Tert-butanol	Polar	0.800	Fair
Diethyl ether	Polar	0.870	Fair
Hexane	Non-polar	3.500	Poor
[BMIM][PF_6]	Hydrophobic	–	Fair
12.5 % [BMIM][PF_6], 37.5 % DMSO, 50 % *tert*-butanol	Polar	–	Good
12.5 % [BMIM][PF_6], 87.5 % DMSO	Polar	–	Good
12.5 % [BMIM][PF_6] 87.5 % *tert*-butanol	Polar	–	Fair

Based on Table-1, glucose showed best solubility in DMSO as reaction media. This relates closely to its low log P value and therefore able to dissolve sugars. Solubility of unprotected sugar is best in highly polar solvent, making them an essential component of the solvent system used in this reaction[13]. Fully soluble sugar (aqueous form) in DMSO would lead to a better interaction with the added base (propylamine) compared to the crystalline form sugar.

In spite of this good effect of DMSO, studies showed that high percentage of DMSO usage in an enzymatic reaction could cause stripping of water from enzyme surface and cause unusual unfolding of enzyme which would eventually led to enzyme malfunction[14,15]. Therefore controlled amount of DMSO had to be used in this work.

As the value of log P for polar solvents increase from acetone to diethyl ether, the solubility rate of sugar reduces. Whereas for non-polar solvent such as hexane, the solubility of glucose is relatively poor. Ionic liquid useful in most enzymatic research due to its excellent stability and activity of lipases[16]. Apart from that ionic liquids also possess highly enantioselective property in single amino sugar synthesis[11] and correlate with 'designer's solvent' tag. Ionic liquid [BMIM][PF_6] possess hydrophobic nature, which is very important for this condensation reaction as presence of water can cause reversible reaction in amino sugar synthesis.

Cao *et al.*[8] workers have reported on *tert*-butanol being a good solvent for optimum lipase activity. Understanding the importance of DMSO, *tert*-butanol and ionic liquid [BMIM][PF_6], mixture of solvent evaluated in this research as reaction media. Mixture of solvents in amino sugar synthesis is based on 'green chemistry' to use reduced amount of organic solvent, high sugar solubility and high product yield motives.

Enzyme screening: Lipase as a multipurpose enzyme chosen to be catalyst in this enzymatic synthesis based on its ability to catalyse aminolysis reactions[17]. Out of 4 enzymes screened, immobilized *Candida antarctica* or most commonly known as Novozyme 435 showed the best conversion (10.68 %) whereas immobilized lipase *Rhizomucor miehei* (Lipozyme RM IM) showed lowest conversion (3.57 %) level (Fig. 1).

Best conversion achieved using immobilized *Candida antarctica* lipase may be due to its support which tolerates highly polar solvent and high temperature resistant[18]. This finding is in agreement with the report Sheldon[19] by stating that the conversion rate is higher using immobilized *Candida*

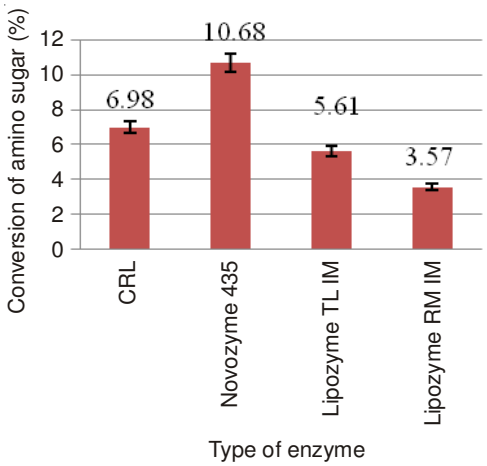


Fig. 1. Screening of enzyme at 50 ºC, 5 mg enzyme mass, 1:1 molar ratio (glucose:propylamine) for 2 h

antarctica lipase compared to free enzyme as biocatalyst with ionic liquid as reaction solvent.

Time course study: Reaction time is a critical point to consider in any chemical processes. Shorter time means highly cost effective condition as a consequence of low energy required for reactions. Our research agreed on the increasing trend of amino sugar product upon increasing the reaction time. This could be due to the increase in reaction kinetics of substrates upon prolonging reaction period leading to higher possibility of molecular collision to produce amino sugar.

At reaction duration of 0.5 h, conversion of amino sugar was 0.15 %. Increment of reaction time to 1 h and 2 h showed 5.77 and 5.85 % amino sugar conversion, respectively (Fig. 2). Further increment in reaction time to 4 h gave low yield (0.73 %) due to the reversible nature of condensation reaction scheme which causes hydration to occur in the substrate favouring direction. Optimum reaction duration for amino sugar derivative synthesis falls in the range of 1 to 2 h.

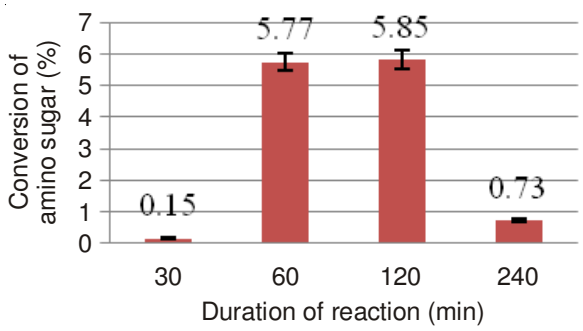


Fig. 2. Synthesis of amino sugar for varying reaction duration, at 50 ºC, 1:1 molar ratio (glucose:propylamine) and 5 mg Novozyme 435

Study on reaction temperature study: Temperature effects on this enzymatic synthesis are well portrayed in Fig. 4 using three different temperatures. The optimum reaction temperature for this work revolves around temperature of 40 ºC with high percentage of yield (8.90 %) in comparison to other temperatures tested. Temperature of 30 ºC was not favoured in this reaction as a classic example of low reaction kinetics.

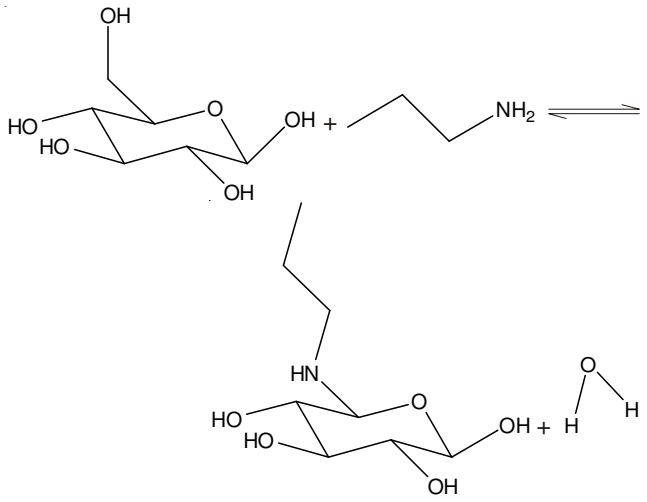


Fig. 3. Synthesis of amino sugar

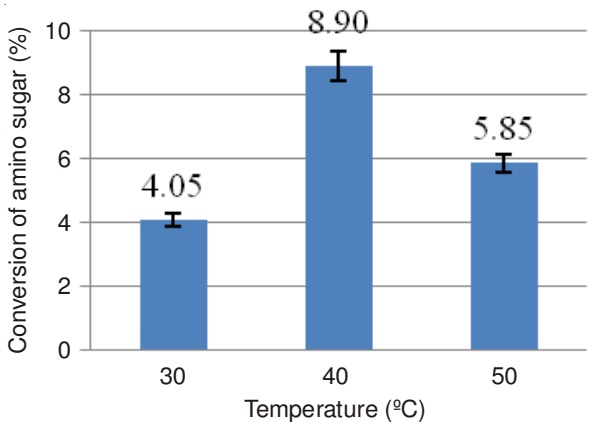


Fig. 4. Amino sugar synthesis at varying reaction temperature, 5 mg novozyme 435, 1:1 molar ratio (glucose: propylamine), for 2 h reaction time

In contrast to finding by Abdul Rahman *et al.*[20], on the stability of Novozyme 435 up to 50.7 ºC, amino sugar showed a decrease in yield at 50 ºC with 5.85 % conversion compare to 8.90 % conversion at 40 ºC. Propylamine's high volatility property at temperature above 48 ºC contributes to this and causing low conversion.

High temperature certainly corresponds to high kinetic energy of reactants and therefore correlates to high collision frequency leading to higher rate of amino sugar formation. Boiling point of propylamine indicates the suitability of propylamine to be used in reactions below its boiling point. Above 50 ºC, propylamine molecules vaporized from bottom of the vial to reside on the vial's wall which is much cooler.

Molar ratio study: Amount of substrate is an extremely important element to study in this reaction to obtain high percentage of yield. Sharma *et al.*[21] revealed that the increase in secondary-amide surfactant conversion from 58.8 to 96.2 % upon increasing ester to amine ratio 1:2 to 1:4. This research showed a molar ratio of 1:5 mmol (glucose: propylamine) was the optimum substrate level to give high percentage (71.56 %) of product (Fig. 5). There was a drastic improvement in conversion level compared to conversion using 1:1 molar ratio of substrates.

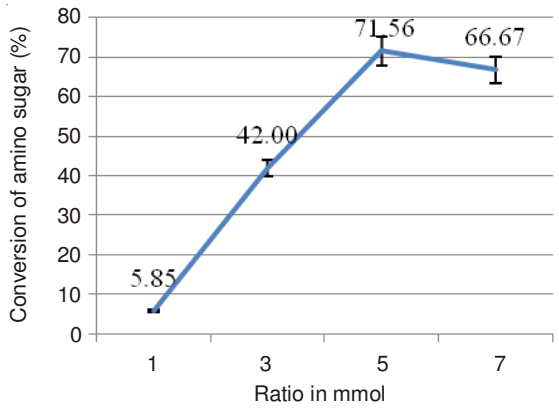


Fig. 5. Synthesis of amino sugar using various molar ratio of glucose to propylamine, using 5 mg of novozyme 435, at 50 ºC for 2 h

Further increment in amount of propylamine (1:7 molar ratio) gave a negative effect on percentage of yield due to substrate limiting factor. Above the optimum molar ratio value, the amount of glucose readily available for alkylation reduces. Therefore the percentage of conversion drops slightly to 66.67 %.

Enzyme quantity study: The substrate concentration should be as high as possible to obtain high degree of alkylation of the sugar used. Simultaneously the amount of immobilized enzyme used should be low as much it is possible to obtain the desired product[22]. Best conversion of amino sugar was obtained upon 2 mg enzyme mass usage (Fig. 6).

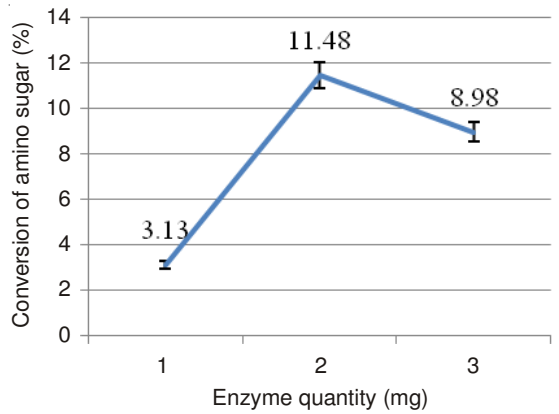


Fig. 6. Synthesis of amino sugar using differing amount of novozyme 435 mass, using 1:1 molar ratio at 50 ºC for a duration of 2 h

Previous work by Kwon *et al.*[23] on synthesizing (S)-naproxen ethyl ester in supercritical carbon dioxide also agreed with the increasing pattern in amino sugar conversion upon providing more enzymes for this synthesis[23]. Low biocatalyst active sites using 1 mg of enzyme mass results in low level of amino sugar conversion. Increment of 8.35 % of conversion occurred upon increasing the amount of enzyme two fold followed by a decrease in conversion percentage (8.98 %) upon increment in enzyme amount three fold.

Similar finding were reported previously by Radzi *et al.*[24]. This is due to the amount of substrate acting as limiting factor in proceeding this enzymatic reaction towards product's favour. Unchanged substrate amount gives a condition whereby substrate molecules saturated with enzyme causing further enzyme addition have no free substrate molecules for reaction.

Optimum condition reaction: An optimum condition in glucose amino sugar synthesis is then used for optimization study. Optimized condition gave 73.37 % conversion of product. This corresponds with the optimum time parameter (2 h), (0.5 h), enzyme mass (2 mg), molar ratio 1:5 (glucose: propylamine) at a temperature of 40 ºC (Fig. 7). Understanding the nature of commercialization for low energy and cost input and to yield effective high output, these optimum conditions well cruises into commercializing pathway. However the yield is not at maximum level due to solvent factor which is the most relevant topic to talk on in this enzymatic facile synthesis.

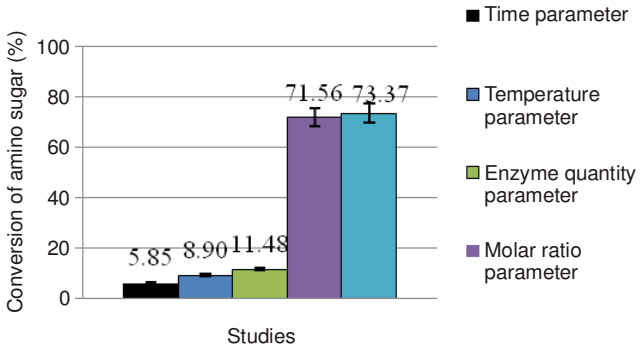
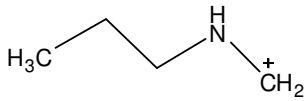


Fig. 7. Optimization study on amino sugar synthesis

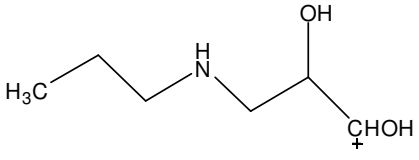
Usage of highly polar solvent in this enzymatic synthesis could cause denaturing of enzyme. Dimethyl sulfoxide is an important element in glycotechnology to provide homogeneity in substrate phases to enable for greater mass transfer[5].

Gas chromatography mass spectrometry analysis of glucose amino sugar: Analysis using GCMS gave few important fragments of amino sugar which corresponded to the success of this research. N-Methylenepropan-1-aminium (Fig. 8) was a fragment of amino sugar (Fig. 3) with mass to charge ratio value 72, which was detected using GCMS. Another amino sugar fragment with mass to charge ratio value 132 as shown in Fig. 9, was also detected. These compounds relate to the existence of reaction between propylamine and glucose molecules as they form the C-N bond.



N-methylenepropan-1-aminium
Chemical Formula: $C_4H_{10}N^+$

Fig. 8. Amino sugar fragment at mass to charge ratio 72



1,2-dihydroxy-3-(propylamino)propan-1-ylium
Chemical Formula: $C_6H_{14}NO_2^+$

Fig. 9. Amino sugar fragment at mass to charge ratio 132

Conclusion

Amino sugar derivative was synthesized in this research. Contrast to the usual chemical hydrolysis method used to synthesize amino sugar derivatives, this research focused on amino sugar derivative synthesis by means of glucose and bases. The reaction media for this enzymatic synthesis is mixture of ionic liquid [PF_6], DMSO and *tert*-butanol. This work revolves around greener and safer material design in order to support sustainable chemical synthesis approach.

ACKNOWLEDGEMENTS

This project was financially supported by research grants from Genetics and Molecular Biology Initiative, Ministry of Higher Education (MOHE), Malaysia and Universiti Putra Malaysia (UPM).

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