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Entrapment of Glucose Oxidase in Reverse Micelle Microemulsion Systems for Glucose Detection in Lipid Based Food Products

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Entrapment of glucose oxidase (GO_x) enzyme in a new reverse micelle emulsion system was studied. The microemulsion consists of aqueous phase (buffered enzyme)/SPAN 85/*n*-decane. Critical micelle concentration (CMC) of surfactant-SPAN 85 in *n*-decane was determined using dynamic light scattering study and it was used to develop microemulsion system. Most stable and optically transparent microemulsion with entrapped glucose oxidase showed higher values of specific enzyme activity, maximum reaction rate (V_{max}) and turn over number and low value of Michaelis-constant (K_m) in comparison to homogeneous GO_x (enzyme-glucose oxidase) system. The microemulsion system was successfully used to quantify D-glucose in lipid based food products without any sample preparation. Comparison of these results with chemical method (phenol-sulfuric acid method) and commercial kit method used in food industry validate the efficiency of the new proposed system. The study provides new information about the glucose content of some commonly consumed milk based products where nutritional labels do not accurately show true glucose content. These findings provide support for comprehensive glucose labeling to food products commonly used by the children.

Keywords: Enzyme kinetics, Food, Microemulsion, Reverse micelle.

INTRODUCTION

Food has straight impact on our health. Nowadays, manufacturers of all packed foods and beverages declare their nutritional content and calorific value, where carbohydrate content is of great significance due to increasing problem of diabetes in the world [1]. The tremendous increase in incident of diabetes coincides with the radical changes of food habits. The continuing interest in opportunities for the personalized prevention and management of diabetes require information of nutritional content in the food. US Food and Drug Administration (FDA) has updated the nutrition facts label requirements where all nutritional labels on food packages will have to provide information on the amount of added sugar by 2019 [2]. Similarly, in India Food Safety and Standard Authority of India (FSSAI) has guidelines for nutritional labels used in food market [3]. In packaged foods and beverages, nutritional information about carbohydrate contents is denoted as total carbohydrate and/or carbohydrate and sugars. The amount and composition of sugars play a decisive role in the sensory properties and acceptability of beverages specifically [4,5]. Regular beverages are sweetened with cane or beet sugar, high fructose corn syrup or blends of these sweeteners. These sugars result in a mixture of sucrose and/or fructose and glucose in the final product which has direct impact on the blood glucose profile of the consumer. There are many kind of food products that are formulated with different recipes but the package display different ingredients. Thus, it is essential to analyze the ingredient of food samples, especially glucose for monitoring the quality of product, determine the contaminants or verify the authenticity of products and its use.

Conventional methods for quality control and food safety involve analytical techniques that are expensive and require specific equipment and trained people. Official methods for measuring glucose in different non-aqueous or lipid based products like milk powder, milk based drinks, vegetable oils *etc*. include chemical, volumetric and polarimetric methods, and gas and anion exchange chromatographic methods [6,7] which involve tedious sample preparation step.

Natural enzymes-glucose oxidase (GO_x) as biological catalysts possess remarkable advantages such as high substrate

specificities and high efficiency under mild conditions. It is used to measure blood glucose level with the system glucose oxidase (GO_x)/peroxidase (PO_x)/*o*-dianisidine. But this simple enzymatic approach is seldom applied to quasi water free or non-aqueous system or lipid containing food products owing to the complex nature of the food samples containing various water insoluble components which may interfere with the glucose measurement. Thus, sample preparation is a pre-requisite for testing glucose in food samples. For this reason, developing a universal detection and quantification method for glucose in quasi water free or non-aqueous system or lipid based systems has great significance in food analysis [8].

GO_x is a hydrophilic enzyme. Thus, immobilization is often the key to optimizing the operational performance of an enzyme, especially for use in non-conventional media. One of the easiest and effective methods for regulating enzyme activity in non-conventional media is the enzyme entrapment within water-in-oil (W/O) microemulsions. Enzyme activity of GO_x has been reported in various reverse micellar system [9,10]. W/O microemulsions, comprising of water, oil and surfactant forming a single optically isotropic, low-viscosity and thermodynamically stable solution, have been used as an attractive approach for enzyme immobilization to facilitate enzymatic catalysis in non-conventional media, because of their long-term stabilities [11-13]. In these systems, the water phase is dispersed in the organic solvent as tiny droplets surrounded by a monolayer of surfactant molecules, forming the so-called reverse micelles [14]. The study of proteins entrapped in such micro-heterogeneous environments has attracted increasing interest due to their use as model systems for studying enzymatic reactions and their potential application in biotechnology [15-17].

The aim of present study is to entrap glucose oxidase from *A. niger* in aqueous droplets of W/O microemulsion containing hydrophobic, biodegradable, food grade surfactant SPAN 85 which can be used in non-conventional medium for glucose analysis (free sugar) present in lipid containing food products. In food industry GO_x is used to remove glucose from dried egg [18] and oxygen from fruit juices and canned beverages [19]. But it is seldom used to measure glucose (free sugar) present in milk based emulsified products like milk powder, milk based drinks, vegetable oils, *etc*., which has great impact on our health. We selected SPAN 85 surfactant having low value of hydrophile-lipophile balance (HLB) and large molecular mass which has a high partition coefficient to organic phase like *n*-decane and stabilizes reverse micelle in organic phase. This gives rise to new possibilities for the universal use of the method in emulsified systems with no sample preparation step.

EXPERIMENTAL

Sorbitan trioleate (SPAN 85), (m.w. 957.49 g/mol, HLB = 1.8 ± 1.1), glucose oxidase (GO_x), (EC 1.1.3.4, 250 U/mg, from *A. niger*) were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), India and *n*-decane was bought from Spectrochem, India. Phosphate buffer and acetate buffers were procured from Merck specialties Pvt. Ltd. All other chemicals were purchased from Fisher Scientific, India. All the chemicals were used as received without any further purification. All experiments were performed using double distilled water. For spectroscopic studies UV-visible spectrophotometer of Ocean Optics DH-mini was used. Hydrodynamic diameter was determined using dynamic light scattering (DLS) technique of Malvern instruments (Zetasizer Nano S90) equipped with He-Ne laser of 4 mW adjusted at 633 nm of wavelength. Detector receives light scattered at an angle of 90º.

Characterization of reverse micelle and W/O emulsion using dynamic light scattering (DLS) technique: The reverse micellar system composed of *n*-decane and SPAN 85, was prepared by adding different concentration of SPAN 85 ranging from 1-15 % (w/w) in 1 mL of organic phase and allowed to stir at 500 rpm for 8 h with uniform mixing. Hydrodynamic diameters of reverse micelles so obtained was measured at 25 ± 5 ºC using DLS method.

Aqueous phase consisting of GO_x, horse radish peroxidase (HRP) and *o*-dianisidine in phosphate-acetate buffer (100 mM, 7.0 pH) was added to a vial containing reverse micelle (*n*-decane and SPAN 85) under constant stirring at 500 rpm. Various phases appear (transparent to opaque) during the addition of different volume fractions of aqueous phase. For each distinct phase, 1 mL aliquot of sample was placed in a quartz cuvette and analyzed using DLS to estimate the mean hydrodynamic size and the polydispersity index (PDI) of emulsion droplets.

Stability of optically transparent W/O microemulsions was checked by measuring the hydrodynamic diameters of aqueous phase droplet for four weeks.

Enzyme assay and steady-state kinetics: The most stable and optically transparent microemulsion (aqueous phase containing GO_x, HRP and *o*-dianisidine in buffer/SPAN-85/*n*-decane) was used for the measurement of enzyme activity. The final concentration of the substrates are: 100 µg *o*-dianisidine, 10 µg GO_x and 5 µg HRP referring to 1 mL micellar solution, respectively. The reaction kinetics was studied using, 5 µL substrate solution (glucose of appropriate mass, dissolved in microemulsion containing 0.1 M phosphate-acetate buffer, pH 7). Enzyme activity of homogeneous GO_x solution in aqueous buffer system and entrapped GO_x in microemulsion containing aqueous buffer/SPAN 85/*n*-decane were determined separately on the basis of oxidation of *o*-dianisidine by glucose oxidase/peroxidase coupled system as reported [20,21]. Absorbance was measured at 460 nm as a function of time for determining reaction rate. Enzyme assay was performed at different glucose concentration varying from 18 to 1800 µg/mL at 25 ± 5 ºC and pH 7.0 to determine parameters like V_{max}, K_m and turnover number (K_{cat}).

Effect of aqueous phase buffer pH was studied by measuring enzyme activity at different pH values of aqueous phase phosphate-acetate buffer solution measured by combined glass pH electrode at 25 ± 5 ºC. Similarly, enzyme stability in microemulsion system was checked by measuring enzyme activity of GO_x at different time interval for four weeks and compared it with the homogeneous aqueous GO_x solution. All the solutions were stored in refrigerator at 4 ºC and brought to 25 ± 5 ºC before measurement of enzyme activity. Percent residual activity was calculated and plotted against number of days.

The percent immobilization for entrapped GO_x was calculated as follows:

$$\text{Immobilization (\%)} = \frac{\text{Specific activity of etrapped } GO_x}{\text{Specific activity of soluble } GO_x} \times 100$$

The residual activity (%) is calculated for soluble GO_x and entrapped GO_x as follows:

$$\text{Residual activity (\%)} = \frac{\text{Observed activity } (GO_x)}{\text{Initial activity } (GO_x)} \times 100$$

Estimation of glucose in food by chemical method: Phenol-sulphuric acid method is the standard chemical method for determining total carbohydrate in different food samples. Here, it is used to determine D-glucose in food samples. [22]. In short, 2 mL of D-glucose solution of appropriate concentration is pipetted into a test tube and 0.05 mL of 80 % phenol is added to it. Then 5 mL of concentrated sulfuric acid is added rapidly, directly on the surface of solution to get proper mixing. The tubes were vortexed twice at an interval of 20 min and then placed in a water bath at 25-30 ºC. The absorbance was measured at 490 nm wavelength. Blank solution was prepared for reference by substituting glucose solution with distilled water. A calibration graph is obtained by plotting absorbance recorded at different glucose concentration.

Estimation of glucose in food by commercial kit method: Commercial-kit method from Megazyme-2018 was used to measure glucose as per manufacturer′s instruction. Calibration curve was obtained with D-glucose as standard. It is used to estimate glucose in food samples. Blank solution is prepared for reference by substituting glucose solution with distilled water.

Estimation of glucose in food by proposed method: A calibration graph was obtained at fixed enzyme concentration by plotting absorbance recorded at different glucose concentration. Appropriate emulsion was taken as blank reference. It is used to estimate glucose in food samples. For coloured food samples, blank solution was prepared for reference by substituting coloured solution with distilled water or microemulsion as needed. Sugars are sensitive to heat and acid hydrolysis of polymers under mild conditions. Thus heat and acid conditions are prevented which may cause changes in the monosaccharide content of sugars. Thus, neutral buffer is used in present experiment.

RESULTS AND DISCUSSION

Encapsulation of GO_x in microemulsion: There is enriched literature available on microemulsion based enzyme immobilization in non-conventional media [23,24], which depicts the hydrophilic reversed micellar inner core as micro-water pool apt for entrapment of hydrophilic enzyme/proteins used in various biotechnological applications. Usually, ionic micellar systems are used for extraction of enzyme, wherein the electrostatic attraction between the inner micellar charge wall and the biomolecules is the main driving force for the transfer of biomolecules from a bulk aqueous phase to polar core of reversed micelles [9,25,26]. However, the recovery yield and the separation factor of an ionic reversed micellar extraction are usually low because of the intensive electrostatic interactions and the low selectivity of electrostatic interactions [27]. Furthermore, the intensive electrostatic interactions often lead to enzyme inactivation or protein denaturation resulting from protein unfolding [28-30]. Therefore, for enzyme catalysis applications we chose food-grade non-ionic surfactant (SPAN 85) for the entrapment of GO_x with HLB value less than 7 which promotes W/O emulsions [31]. Here non-ionic surfactant shows less pronounced interactions with the enzyme molecules entrapped in the non-ionic hydrophilic core of reverse micelle with minimal effect on its structure, activity/property. We have chosen *n*-decane as the oil phase because of its low dielectric constant (ε ~ 1.991) and non-volatile nature at room temperature which prevents material loss over the course of our experiments.

It is also desirable to form stable emulsions/microemulsions with a minimum amount of surfactant. The critical micelle concentration (CMC) of surfactants for aqueous-surfactant systems has been well-studied, but few data are available for organic-surfactant systems. Hence, we employed DLS measurements to study the reverse micelle in the organic phase, which allows measurement of hydrodynamic size and provides information on the surfactant′s CMC as reported [32-34]. SPAN 85 molecules have highly branched structure which forms relatively larger microstructure in *n*-decane as reverse micelles. The DLS measurement results obtained for SPAN-85 in *n*-decane are shown in Fig. 1. Each data point is averaged from three independent measurements and the standard deviation is calculated. The hydrodynamic diameter of SPAN-85 aggregates in *n*-decane initially showed no gradation up to 22.5 mM of surfactant concentration and PDI value (0.3-0.8), then reveres micelle size increases and attains a roughly constant value of 252 ± 4.75 nm over a wide range of concentration. It confirms that SPAN 85 forms stable micellar-type aggregates at concentrations of 62.5 mM or above.

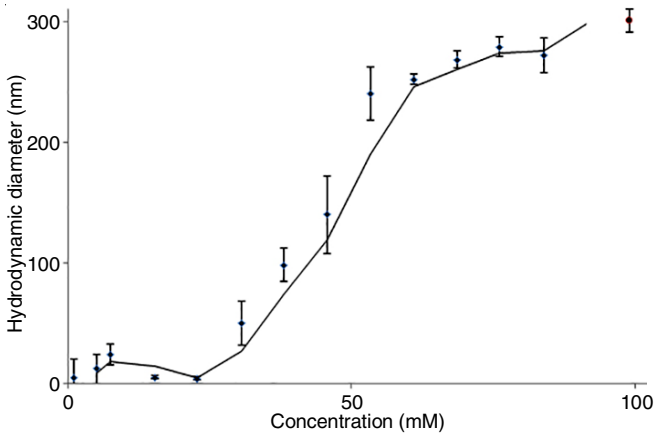

Fig. 1. Apparent hydrodynamic diameter of SPAN 85 aggregates in *n*-decane measured with DLS

Water is fundamental for enzyme activity. In W/O-type emulsions at the CMC and above, the spherical micelles enclose the aqueous phase in so-called water pools [33,35], where the size of the pool depends on the relative quantity of the aqueous phase as well as the number of surfactant molecules forming the micelles. From practical point of view, glucose assay requires GO_x along with HRP, glucose and *o*-dianisidine dye for spectrophotometric measurement which are water soluble. Thus, in present system, aqueous phase consists of GO_x, HRP and *o*-dianisidine in phosphate-acetate buffer. Reasonably satisfactory aqueous phase/SPAN85/*n*-decane emulsions over a wide range of aqueous phase volume fractions could be prepared. Since buffer solution with enzymes is insoluble in oil (*n*-decane), the GO_x is confined within the aqueous boundary. Optically transparent reverse micelle system are produced by dispersion

of small volume of aqueous phase into oil phase in presence of fixed amount of surfactant known as Winsor IV system [36] and thus enzymes entrapped in a space of low water content in Winsor IV system is suitable for spectroscopic studies. Thus, different amount of aqueous phase was added in reverse micelle containing 62.5mM SPAN 85 in 1.0 mL *n*-decane to obtain emulsions and hydrodynamic diameter of swollen reverse micelle was measured by DLS technique. Fig. 2 shows that the size of spherical aqueous droplet grows with the increase of aqueous content. Increasing the degree of hydration of reverse micelle at fixed enzyme concentration leads to the increase in size of inner cavity in reverse micelle system. At constant surfactant: *n*-decane ratio and low aqueous phase concentration region the microemulsion was optically transparent. If the volume of aqueous phase increases beyond 50 µL, the microemulsion become slightly turbid/translucent to milky appearance as shown in Fig. 3, where bottle A represents Winsor IV system with the droplet size 280.5 ± 9.2 nm, bottle B represents translucent system with droplet size 424.8 ± 9.9 nm and bottle C represents turbid milky system with droplet size 602.7 ± 6.9 nm, respectively (corresponding DLS data is given in supporting information file). Similar observations are also reported for anionic surfactant (AOT) in isooctane [37]. Molar ratios of water solubilized in a reverse micelle emulsions (W_o) are important parameter which has been extensively studied to determine the enzymes hydration rate and its activity. But entrapment of GO_x/HRP/*o*-dianisidine in reverse micelle is not exactly immobilization as we observed on solid support. The assemblies are dynamic species and inter-micellar exchange of molecules including proteins is quite fast [38,39] and it causes a rearrangement of reverse micellar aggregates and affect the equilibrium of the system [40]. These systems may show instability like phase separation, sedimentation or creaming due to coalescence and Ostwald's ripening which will affect their application in enzyme catalysis [41]. Thus, we determined the stability at maximum hydration of reverse micelle where W/O microemulsion is optically transparent. Fig. 4 shows the corresponding stability of aqueous droplets in the swollen micelle. The stable microemulsion with aqueous droplet size 280.5 ± 9.2 nm do not show any appreciable change in their size are suitable for enzyme kinetics study.

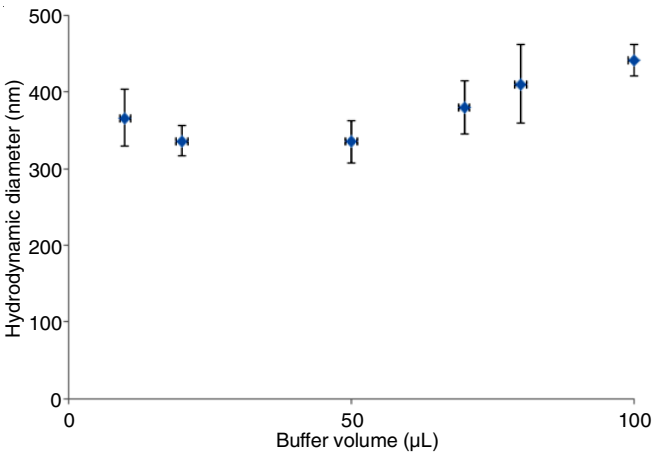

Fig. 2. Variation of W/O microemulsion droplet size as a function of aqueous phase volume containing constant

Fig. 3. W/O emulsion representing A: Optically transparent microemulsion; B: slightly turbid emulsion; and C: milky appearance-emulsion obtained by different ratio of Aqueous phase : SPAN 85 : *n*-decane

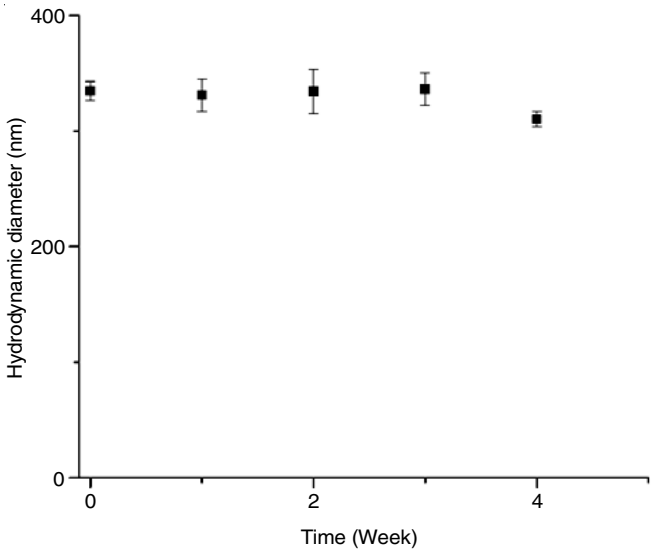

Fig. 4. Stability of aqueous phase droplet containing GO_X and constant surfactant:*n*-decane ratio as a function of time in week

Enzyme kinetics and glucose determination: Water-in-oil is thermodynamically stable single phase dispersions where hyperactivity of HRP and many other enzymes are reported [10,42-45]. In general, the biochemical reaction catalyzed by enzymes in reverse micellar system follows same kinetic behaviour as in homogeneous system. In the present study, kinetic analysis was carried out according to the classical Michaelis-Menten equation:

$$v = \frac{k_2[E]_0[S]_0}{K_M + [S]_0} = \frac{V_{max}[S]_0}{K_M + [S]_0} \quad (1)$$

where, $[E]_o$ and $[S]_o$ are GO_x and glucose molar concentrations, respectively, k_2 is the first-order catalytic constant, K_M is the Michaelis constant; and $V_{max} = k_2[E]_o$ is the maximum rate realized at saturation of enzyme with substrate. Keeping overall concentration of GO_x, HRP, *o*-dianisidine constant, the analysis of GO_x reaction kinetics was studied at different glucose concentration in W/O microemulsion system in comparison with homogeneous aqueous solution. It allows the determination of maximum reaction rate (V_{max}), inverse measure of the enzyme's affinity for the substrate (K_M), turn-over number (K_{cat}) and catalytic efficiency of GO_x in these systems as shown in Table-1. In the present study, *o*-dianisidine oxidation to give colour in microemulsion system was faster than in homogeneous aqueous

solution at the same over-all glucose and same reagent concentration. The enzyme activity of GO_x in microemulsion system is almost two-fold higher than in homogeneous aqueous solution (Table-1). It is apparent that GO_x is comparatively more active in the present non-ionic micellar system. For lyophilized GO_x in aqueous buffered system K_M amounts to 8.9 mM while it is smaller (6.87 mM) at maximum aqueous phase concentration in reverse micelle microemulsion system showing better affinity of D-glucose towards GO_x in the system. Different models are proposed to explain higher activity of enzymes in W/O microemulsions. Practically, transfer of hydrophilic glucose molecule entrapped in a reverse micelle to swollen micelle containing GO_x controls the rate of reaction.

Homogeneous biochemical reactions are highly sensitive to pH of the buffer solution used as reaction medium. Strategies to study the effective pH of water core inside the swollen reverse micelle are not standard. Thus, we determined the GO_x activity as the function of pH of aqueous phase buffer solution used in present study to develop microemulsion. The pH optimum for homogeneous solution of GO_x is 5.5 but for microemulsion at 25 ºC, enzyme activity was almost constant between pH 5.5-7 as shown in Fig. 5. At present, it is not possible to explain this effect. Mukherjee *et al*. [46] had reported that a proton gradient exists inside the water-pool of charged reverse micelles. Possibly, in our case the reverse micelle is non-ionic in nature with non-polarizable groups, thus no proton gradient is present which may affect the enzyme activity. Hence, pH of buffer forming aqueous phase do not affect the over-all GO_x reaction.

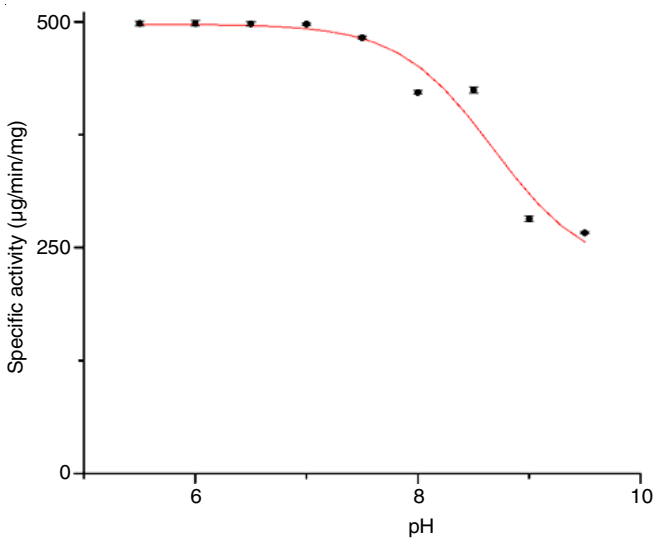

Fig. 5. Dependence of GO_X enzyme activity on the pH value of aqueous phase buffer used to develop microemulsion

Storage and stability of GO_x in microemulsion: The microemulsion containing enzymes, *o*-dianisidine and homogeneous aqueous solution were stored at 4 ºC for 4 weeks and enzyme activity of GO_x was determined at 25 ºC. Residual enzyme activity was calculated. Fig. 6 shows the variation of percent residual GO_x activity in microemulsion system in comparison to the aqueous homogenous system. The enzyme activity is more than 90 % for entrapped GO_x confirms its suitability for storage in bottles.

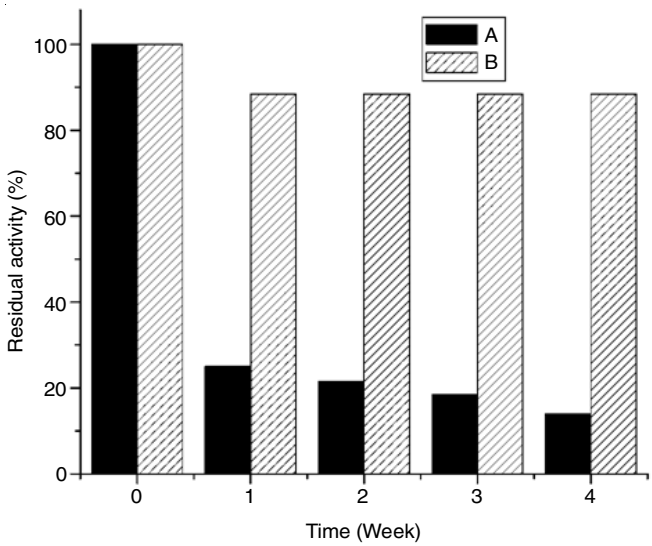

Fig. 6. Stability of GO_X: (A) in phosphate-acetate buffer and (B) entrapped in W/O microemulsion over a period of 4 weeks

Estimation of glucose in different food products: The microemulsion system containing GO_x-HRP enzyme coupled with *o*-dianisidine was applied to the analysis of lipid containing food stuffs like commercial milk based drinks, oil and beer. For most lipid containing foods, sample preparation is an important step where water soluble carbohydrates like D-glucose measurement using commercial enzyme based kit can not be achieved without extraction of lipids and lipid soluble substances. Here, D-glucose content was directly analyzed in five replicates without any specific sample preparation step. The results were compared with the colorimetric method used for total carbohydrate determination [22] and glucose determination by commercial glucose assay kit for its validity. W/O microemulsion consists of aqueous phase dispersed in oil phase where hydrophobic components like lipid molecules or lipid soluble molecules can diffuse in oil phase and hydrophilic components can move to aqueous phase by diffusion. Thus, it is assumed that the lipid molecules from food products were dissolved in the oil phase and do not interfere with the glucose and enzymes present in the aqueous phase. Calibration graph was obtained by using D-glucose as standard for present W/O microemulsion method as shown in Fig. 7. It has a good linear calibration range which can detect glucose from 18 to 900 µg/mL of D-glucose sample with a good linear regression coefficient (R^2) of 0.9926. Table-2 shows the amount of glucose measured

TABLE-1
KINETICS AND STABILITY PARAMETERS OF GO_X ENZYME IN AQUEOUS BUFFER SOLUTION AND ENTRAPPED W/O MICROEMULSION

Enzyme condition	Specific activity (µmol/min/mg)	Immobilization (%)	V_{max} (mM/min)	K_m (mM)	Turn over No. (s^{-1})	Storage and stability with 90 % activity (days)
Aqueous GO_X	236.36	–	0.78	8.9	6.2×10^5	7
Immobilized GO_X	475.75	201.28	1.57	6.87	1.3×10^6	30

TABLE-2
GLUCOSE ASSAY OF DIFFERENT FOOD PRODUCTS WITH DIFFERENT METHOD FOR THE DETERMINATION OF CARBOHYDRATE AND GLUCOSE CONTENT

Sample name	Ingredients		Nature of sample	Additives	Methods		
	Carbohydrate content	Sugar/glucose			A (Total carbohydrate as glucose)	B (Only glucose)	C (Only glucose)
Toned milk	5.1 g/100 mL	$	Emulsified mixture	$	4.6 ± 0.25 g/100 mL	1.3 ± 0.01 g/100 mL	1.5 ± 0.25 g/100 mL
Soya milk	24.6 g/200 mL	Sugar = 21.0 g/200 mL; Glucose = $	Emulsified mixture	Contains added flavour	21.2 ± 1.17 g/200 mL	0.7 ± 0.14 g/100 mL	0.6 ± 0.06 g/100 mL
Milk powder	59.8 g/100 g	Sugar = 57.6 g/100 g; Glucose = $	Nature of sample is Powder made into solution as per manufacturer instruction	Contains stabilizer (339 (iii))	55.4 ± 0.29 g/100 g	2.8 ± 4.4 g/100 g	2.6 ± 0.6 g/100 g
Horlicks	78 g/100 g	Sugar = 32 g/100 g; Glucose = $	Nature of sample is Powder made into solution as per manufacturer instruction	Contains permitted natural colour and added flavour	63.5 ± 2.5 g/100 g	3.4 ± 0.79 g/100 g	2.8 ± 0.2 g/100 g
Lager beer	11 g/100 mL	Sugar = 0.0 g/100 mL; Glucose = $	Alcoholic	No preservative and flavor added	7.4 ± 1.9 g/100 mL	0.05 ± 0.03 g/100 mL	0.09 ± 0.02 g/100 mL
Premium beer	Not mentioned	$	Alcoholic	$	4.5 ± 2.7 g/100 mL	0.04 ± 0.01 g/100 mL	0.14 ± 0.06 g/100 mL
Olive oil	0 g/100 g	Sugar = 0 g/100 g; Glucose = $	Emulsified mixture	$	7.4 ± 2.17 g/100 g	0.06 ± 0.02 g/100 g	0.08 ± 0.001 g/100 g
Rice bran oil	0 g/100 g	$	Emulsified mixture	$	32.7 ± 0.56 g/100 g	0.11 ± 0.02 g/100 g	0.14 ± 0.01 g/100 g

*$ = Not labeled; A = Phenol-Sulfuric acid method for total carbohydrate determination, B = Enzymatic glucose determination by commercial kit and C = Present method developed in our lab

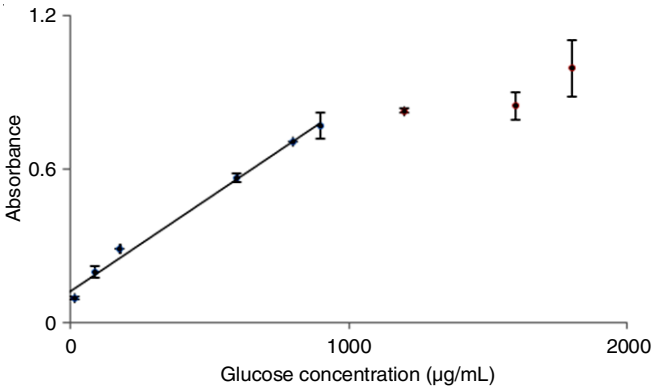

Fig. 7. Calibration plot of glucose determination obtained by present W/O microemulsion method

with chemical method (phenol/sulfuric acid reaction system) [22], glucose measured by commercial kit (Megazyme GOPOD enzyme assay) and present W/O microemulsion method. As can be seen from Table-2, there is a good agreement between the results obtained with W/O microemulsion based method and the reference photometric kit. It is interesting to observe that the results obtained with the reference spectrophotometric kit all show slightly higher values, probably because of the multienzyme cascade reactions, which take place in the kit and that can cause interferences from other compounds, especially other sugars, whereas the microemulsion system based method is strictly specific for glucose as demonstrated above.

Conclusion

The glucose oxidase activity is studied in a new microemulsion system consist of aqueous phase (GO_x/HRP/*o*-dianisidine in phosphate-acetate buffer)/SPAN 85/*n*-decane where SPAN 85 in *n*-decane forms stable reverse micelle at CMC of 62.5 mM and above with hydrodynamic size of 252 ± 4.75 nm. Addition of aqueous phase (5-50 µL) to the stable reverse micelle system gives swollen micelles which is optically transparent W/O microemulsion. At aqueous phase concentration of 50 µL and droplet size 280.5 ± 9.2 nm, W/O microemulsion is stable for 4 weeks. GO_x enzyme activity measured in microemulsion is double in comparison to homogeneous enzyme solution at pH 7 and it is almost constant between pH 5.5-7 of aqueous phase buffer dispersed in microemulsion. The K_m value for GO_x biochemical reaction is 6.87 mM and turnover number is 1.3×10^6 s^{-1} showing high activity of GO_x in microemulsion, practically useful for D-glucose measurement in food samples. It shows a good linear range of glucose detection which can detect glucose from 18 to 900 µg/mL with a good linear regression coefficient (R^2) of 0.9926. It is a new, rapid and easy method for determination of D-glucose in food samples. The preparation of the spectrophotometric kit is very simple, low-cost and quick. It does not require a sample preparation step for liquid samples. The detection method was not influenced by presence of lipid molecules or lipid soluble components in food matrices which can act as possible interferents. This microemulsion system can be developed into enzymatic test-kits with ready to use reagents widely used for the analysis of glucose in food products which will bring huge improvement in the laboratory. The colorimetric method is based on specificity of GO_x for glucose which is measured in a few samples by a simple photometer in a small laboratory and outstanding reagents can be stored for further usage. In big laboratories with automation, the liquid reagents can be directly used on any biochemistry analyzer.

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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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