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Activity, Stability, and Binding Capacity of β -Galactosidase
Immobilized on Electrospun Nylon-6 Fiber Membrane

Deborah Ann Hutchins

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Activity, Stability, and Binding Capacity of β -Galactosidase Immobilized on Electrospun Nylon-6 Fiber Membrane

Deborah Ann Hutchins

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Master of Science

This research explores various immobilized enzyme support materials, including the novel nylon-6 fiber membrane (NFM), observing the increase of surface area and what effect that has on enzyme binding potential. This study also manipulates incubation and reaction conditions and observes what affect that has on activity and stability of β -galactosidase comparing various solid support materials and free enzyme. Nylon-6 fiber membranes were created using the process of electrospinning and were compared with other materials as solid support materials for enzyme binding. The other materials included polyvinylidene fluoride 5 kD nanofiltration dairy membranes, nylon-6 pellets, silica glass beads, and free—dissolved—enzyme. Scanning electron microscopy images exposed the nylon-6 fiber membrane's large amount of surface area which coordinated with greater enzyme activity as compared to the relatively flatter surfaces of the other solid support materials. Enzyme activity was measured spectrophotometrically with the color-changing substrate ortho-Nitrophenyl- β -galactoside.

NFM had greater maximum enzyme binding potential than the other solid supports. Across pH conditions ranging from 3.5 to 6.0., enzyme activity was maintained on the membrane immobilized samples whereas free enzyme did not maintain activity. Altering storage temperature (4, 22, and 50 °C) affected enzyme stability, the ability of the enzyme to maintain activity over time, of free and polyvinylidene fluoride membrane samples. However, nylon-6 fiber membrane samples maintained stability across the varying storage temperatures. Increasing the immobilization solution enzyme concentration above maximum enzyme binding capacity had no significant effect on enzyme stability for membrane immobilized samples. Although, both had lower mean stability than free enzyme by approximately 74% percent.

With further development, β -galactosidase immobilized on nylon-6 fiber membranes, or other membranes, could be used in continuous processing in the dairy industry for a combination of filtration and lactose hydrolysis—creating products reduced in lactose and increased in sweetness with no “added sugars” requirement for a nutrition label and no enzyme listed as final product ingredient.

Keywords: nylon-6, membrane, immobilization, β -galactosidase, stability

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INTRODUCTION

Dairy product manufacturers are constantly searching for ways to add value to cheese and whey co-products to meet changing consumer demands. Membrane filtration is commonly used to increase the value of whey products by separating and isolating various whey proteins and lactose for desired compositions of whey retentate and permeate. Whey permeate is the co-product of whey protein production, and although permeate is more abundant, it can be sold at only a fraction of the price of whey protein retentate due to its relatively low concentration of protein (high value) and high concentration of lactose (low value, minimum of 76% lactose on a dry weight basis) (American Dairy Products Institute, 2020). Lactose is the disaccharide inherently present in dairy products; however, about 70% of the world adult population has some form of lactose intolerance (Elnashar and Hassan, 2014). To make dairy products available to the lactose intolerant population, the enzyme β -galactosidase is used to hydrolyze lactose into its monosaccharide components—glucose and galactose (Amaya-Delgado et al., 2006). β -galactosidase is also commonly employed to produce galactooligosaccharides—probiotic compounds produced from dairy products that result in positive physical functions such as growth or immune and antioxidant function when supplemented in the diet (Skrylonek et al., 2017; Xing et al., 2020). Other potential benefits resulting from β -galactosidase activity include increasing sweetness of products without increasing added sugars because neither naturally present lactose, nor its sweeter hydrolysis products, are required to be listed as “added sugars” on the updated nutrition facts panel (21 CFR 168.122 (d) (1); Illanes et al., 2002).

Although β -galactosidase is commonly used in the dairy industry and has potential for increasing the value of dairy products, lactose hydrolysis processes in industry are primarily single-use methods, wherein the enzyme is not recovered. As catalysts, enzymes can be used

multiple times if immobilized onto a stable surface (Szczodrak, 1999), and would not have to be listed as an ingredient in final products even if used in higher doses (Zhao et al., 2017). However, the term “enzyme-modified” may still be required for labeling. β -galactosidase has been immobilized onto various surfaces (Mateo et al., 2004; Mohamad et al., 2014; Wahba, 2017), but not on the highly fibrous and high surface-to-volume Nylon-6 fiber membrane (**NFM**) (Mason et al., 2018; Bhardwaj and Kundu, 2010). Other membranes, such as those made of polyvinylidene fluoride membrane (**PVDFM**), are commonly used in the dairy industry to filter and isolate components from whey solutions due to the small finger-like tunnels of the membrane. Dairy foods are processed through membranes under various conditions manipulating time, membrane surface area, temperature, and pH among other parameters. These conditions are selected to maintain activity and stability of the enzyme during processing, and to maximize efficiency under the varying environments in production plants. Activity is defined as the rate at which the enzyme converts substrate into product—in this example, lactose into glucose and galactose. Stability is the ability for an enzyme to retain activity over time and over various environmental conditions (Jin et al., 2017). Time potentially could be further saved through enzyme immobilization by combining some filtering and enzyme reaction steps and allowing for continuous processing and by eliminating the enzyme inactivation step (Illanes et al., 2002; Rejikumar and Devi, 2016).

This research explores various solid support materials for the immobilization of β -galactosidase. Enzyme activity and stability was compared across materials in various enzyme concentrations from the immobilization solution, temperatures, and pH conditions.

MATERIALS AND METHODS

Materials and Chemicals

PVDFM, with a pore size of 5 kD, was obtained from Synder Filtration, Inc. (Vacaville, CA, USA). β -galactosidase from *Aspergillus oryzae*, EC 232-864-1 (> 5,000 U/g) was obtained from MP Biomedicals LLC (Solon, OH, USA). All other materials and reagents used were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

Support Materials

Materials were selected for comparing immobilization of β -galactosidase. Silica glass beads (**GB**) were commonly used in past research for immobilization and were included for comparison (Gennari et al., 2018). Electrospun NFM was selected due to its high surface-to-volume ratio as reported in other research by Mason et al. (2016) and Rodríguez et al. (2018) and consequent potential for binding more enzyme to increase enzyme activity. Nylon-6 pellets (**NP**) were included as the pre-electrospun material of NFM, to highlight the transformative effect of electrospinning. PVDFM was selected as a common material used in the dairy industry for industrial milk and whey processing.

Electrospinning Nylon-6 Nanofiber Membranes

NFM were prepared in our laboratory by electrospinning following the method of Bhardwaj and Kundu (2010). Nylon-6 was dissolved into formic acid (64%) (20:80). The nylon-6/formic acid solution was added to a 5 mL plastic syringe which had a 22-gauge blunt tip stainless-steel needle (Covidien LLC, Mansfield, MA, USA). The syringe was connected to a syringe-pump (KdScientific model 111 pump, Holliston, MA, USA) and pumped out horizontally at a rate of 0.001 to 0.002 mL/min. A charge was applied to the polymer solution by connecting a Spellman CZE 1000R power supply (Happauge, NY, USA) to the syringe needle

and a grounded aluminum foil collector plate, covered with a sheet of wax paper, positioned 11 cm from the syringe discharge point (Mason et al., 2016). Membranes were created by deposition on the collector plate over a period of approximately 4 to 8 hours and were later standardized by mass, as described below.

After the Nylon-6 nanofiber membranes were made and peeled off of the temporary wax paper support, they were then cut into 7.9 mm diameter circles using a standard hole punch and temporary cardstock paper backing for support while cutting. NFM were weighed and those of a similar mass were selected for grouped analyses. In order to reduce variation of enzyme binding due to membrane density, groups of NFM were weighed and organized to have a range of 2 mg or less. NFM weighed between 4 and 8 mg.

Immobilizing Enzyme onto Solid Support Materials

Prior to the standard enzyme immobilization procedure, the GB had additional surface activation steps. GB were soaked in acetic acid solution (0.01 M) for 1 h to remove possible surface contaminants (e.g. metal ions). The beads were then submerged into a 6:25 (v:v) solution of 3-aminopropyl triethoxysilane (3-APTES) and toluene and left to incubate at 50 °C for 24 hours in order to activate the GB surface in preparation for enzyme immobilization. GB were rinsed in distilled-deionized water and then followed the same immobilization procedure as the other stable supports as described below (Eskandarloo, 2018; Song et al, 2010).

The process for covalently immobilizing enzyme was taken from Salleh (1982) and adjusted for β -galactosidase in place of glucose isomerase. All stable supports were incubated for 4 hours at ambient temperature in 18.5% glutaraldehyde, 0.36 M borate buffer pH 9.0 (Salleh, 1982; Magro et al., 2019). Then the solid supports were rinsed in distilled-deionized water. Activated solid supports were incubated at ambient temperature for 2 hours in 10%

polyethylenimine (**PEI**) solution. The solid supports were then rinsed and incubated for 20 hours at ambient temperature with agitation in enzyme immobilization solution (0.05 M sodium acetate, pH 5.0, 0.005 M L-cysteine) with varying concentrations of β -galactosidase (10, 50, 100, 500 mg/mL) (Amaya-Delgado et al., 2006). Afterwards, the enzyme immobilized solid supports were rinsed with 2 M NaCl and stored at room temperature for 30 minutes in order to remove adsorbed enzyme from the support (Hosseini et al., 2018). The immobilized enzyme solid supports were stored in 0.05 M acetate buffer pH 5.5 at 4 °C until activity was measured (Amaya-Delgado et al., 2006).

Scanning Electron Microscopy

Images of the stable surfaces were developed using scanning electron microscopy (**SEM**). Scanning electron micrographs were collected on a Thermo Fisher Scientific Apreo C SEM. Samples were mounted on aluminum stubs and coated with Au:Pd (80:20) with a Quorum sputter coater supplied by Electron Microscopy Sciences (**EMS**) prior to observation in the SEM. Images were collected at 5 KV accelerating voltage with a beam current of 50 pA.

Enzyme Activity

Activity of β -galactosidase was measured using o-nitrophenyl- β -D-galactopyranoside (**ONPG**) as the substrate. Enzyme activity was defined as the amount of enzyme that liberated 1 mM of o-nitrophenol (**ONP**) per minute under the standard assay conditions; i.e., 50 °C pH 5.5 (Fai et al., 2017; Katrolia et al., 2018). An extinction coefficient for ONP of 537 M⁻¹cm⁻¹ was calculated and used.

Enzyme activity of free and immobilized β -galactosidase was measured by incubating the sample with 1 mL of ONPG at 25 mM in 50 mM sodium acetate buffer pH 5.5 at 50°C for standard conditions—except for pH variation samples; in which case only the pH was modified.

(Rodríguez et al., 2018). Additional buffers were used for the more extreme pH conditions. For the pH 3.5 buffer mM sodium citrate was added, and for the pH 6.0 buffer 100 mM K₂HPO₄ was added to help the buffering zone extend into the designated pH condition (dos Santos et al., 2017). Aliquots (100 µL) of the reaction mixture were withdrawn every 0, 15, 30, 60, and 90 seconds, after aspiration, and added to 200 µL of 0.2 M sodium borate buffer pH 9.8 to stop the reaction. Absorbance of ONP at 405 nm was measured with a FLUOstar OPTIMA UV-Vis spectrophotometer in a CELLSTAR 96 well plate from BMG Labtech Inc. (Cary, NC, USA) (Singh et al., 2009). Enzymatic assays were performed in triplicate.

Default conditions for immobilized enzyme were 50 mg/mL enzyme in the immobilization solution or 2.5 mg/mL free enzyme, incubated at 50 °C, and stored and reacted in pH 5.5 buffer solutions—unless otherwise specified.

Enzyme Stability

Retention of free and immobilized enzyme activity in buffered solution was measured by incubating the samples in storage buffer solution at the designated pH and temperature conditions, as described above, over the course of 24 hours. Enzymatic activity readings were taken every 2 to 4 hours during stability analysis until hour 12 and then again at hour 24. The time at which the relative activity reached 50% was used for reporting stability of free and immobilized enzyme. For those samples that still had greater than 50% of the enzyme activity at hour 24, percent of activity at hour 24 is reported. When manipulating temperature for stability measurements, various incubation temperatures were selected based on refrigeration (4 °C), ambient (approximately 22 °C), and optimized enzymatic activity (50 °C) temperatures.

Statistics

Enzymatic activity and stability of the various samples were analyzed using an unpaired, unmatched, ordinary one-way ANOVA and Tukey's multiple comparisons test to compare statistical differences ($\alpha = 0.05$) assuming equal standard deviation within a material group. When comparing between materials, standard deviations were not assumed to be equal and therefore an unpaired, unmatched, Brown-Forsythe and Welch ANOVA and Dunnett's T3 multiple comparisons test was used instead, unless otherwise stated. The statistical software program used was Prism8 by GraphPad Prism version 8.4.3 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com. Results are reported as mean $\pm$ standard deviation, $n = 3$.

Research goals and experimental design

The major treatments to be compared were the various stable supports, or the lack thereof. Also compared was how the selection of the support affects activity and stability of the enzyme in various environmental conditions. Maximum active β -galactosidase binding capacity of the stable supports was also analyzed. The stable supports compared were PVDFM, NFM, NP, GB, and free enzyme (no stable support). The varying environmental conditions included varying the concentration of enzyme in the immobilization solution, pH during incubation and reaction, and temperature of incubation. The concentrations of enzyme in the immobilization solution selected for comparison were 10, 50, 100, and 500 mg/mL and were also used for comparison of maximum immobilization capacity. The various pH conditions selected were 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0. Temperatures of analysis were selected to be 4 °C, approximately 22 °C (ambient), and 50 °C.

1. Surfaces were prepared and enzyme immobilized onto the solid support. This included the formation of the nylon-6 fiber membrane via electrospinning.

2. Support materials were analyzed for maximum active β -galactosidase binding capacity.
3. Enzyme activity was compared across materials in various enzyme concentrations from the immobilization solution and pH conditions.
4. Enzyme stability was compared across materials in various enzyme concentrations from the immobilization solution, temperatures, and pH conditions.

Hypothesis

H₀: Varying support materials will have no significant effect on neither activity nor stability of β -galactosidase from *Aspergillus oryzae* when environmental conditions of pH, temperature, or enzyme immobilization concentration are altered as observed with spectrophotometry of ortho-Nitrophenyl- β -galactoside ($P > 0.05$).

H_a: Varying support materials will have a significant effect on activity or stability of β -galactosidase from *Aspergillus oryzae* when environmental conditions of pH, temperature, or enzyme immobilization concentration are altered as observed with spectrophotometry of ortho-Nitrophenyl- β -galactoside ($P < 0.05$).

RESULTS AND DISCUSSION

Surface images were taken to view the physical differences between the stable supports before and after β -galactosidase was immobilized (Figure 1 A-H). The assorted magnification levels in Figure 1 were selected to adequately show the characterization of the various surfaces. The electrospinning of Nylon-6 into NFM created long fibers in various directions as seen in Figure 1 A. Observing NFM and highlight the effect of electrospinning on generating surface area which leads to greater potential for enzyme binding (Figure 1 A and B).

Figure 1 A-H. SEM of support materials before and after enzyme immobilization.

SEM images were selected as a representative image of each materials' surface. Images A-D were the material before enzyme immobilization, and images E-H were the same materials but after enzyme was immobilized. With its long fibers, NFM shows the most surface area when compared to the other support materials. Immobilization of β -galactosidase created a noticeable additional layer on the surface of each material. Scales are provided for relative size comparison. NFM = nylon-6 fiber membrane, NP = nylon pellets, PVDFM = polyvinylidene fluoride membrane, GB = silica glass beads.

Variations in the surface of the materials are apparent. For example, NFM (Figure 1 A) has long fibers the other surfaces (Figure 1 B, C, and D) are relatively flat. Electrospinning increased the surface area of the Nylon-6 taking it from a rough, relatively flat surface to a porous collection of long fibers. However, PVDFM is well known to have finger-like pores in a

cross-sectional view that are not captured in a surface image such as this. Increased surface area may contribute to the potential for increasing enzyme immobilization.

A major difference between before and after enzyme immobilization is that after immobilization, the surfaces appear to have a thick coating (Figure 1 E-H) making the surface smoother for NFM and GB (Figure 1 E and H) and more textured for the other samples—NP and PVDFM (Figure 1 F and G). Some of the fibers from NFM appear to be flattened (Figure 1 E) which could be due to handling the membrane during the immobilization process. In the image of PVDFM (Figure 1 G), small cracking appears in the coating along the edges of the uneven surface—potentially due to dehydrating the samples in preparation for the imaging.

Figure 2. Effect of immobilization solution enzyme concentration on enzyme activity for each supporting material.

The concentration of enzyme available during immobilization was increased, and after immobilization, enzymatic activity was measured in triplicate samples ($n = 3$). Error bars representing 1 standard deviation are displayed. Free, dissolved β -galactosidase, indicated with a “*” symbol, (2.5 mg/mL) was used as a reference. Increasing enzyme concentration during immobilization had an effect on NFM ($P = 0.003$), but had no statistical effect on PVDFM, GB, or NP ($P \geq 0.099$). NFM appears to have the most potential for increasing enzyme concentration creating an effect on measured enzyme activity. Free = free, dissolved enzyme, NFM = nylon-6 fiber membrane, PVDFM = polyvinylidene fluoride membrane, GB = silica glass beads, NP = nylon pellets. Letter codes denote statistical differences within material groups.

The various support materials were compared for their ability to bind active enzyme and to determine maximum binding capacity. It was believed that with more enzyme available for immobilization, more enzyme could be immobilized and would therefore result in a greater enzyme activity. This phenomenon was displayed in the NFM with a 69% increase in activity when available enzyme increased from 10 to 50 mg/mL ($P = 0.003$). However, enzyme activity did not continue to significantly increase when concentration of available enzyme increased above 50 mg/mL. Unlike NFM, the PVDFM, GB, and NP materials did not significantly increase in mean activity with increased enzyme available during immobilization ($P \geq 0.099$). Additionally, the activity of the immobilized enzyme on NFM, even at the lowest enzyme concentration of 10 mg/mL, was greater than all other support materials ($P = 0.001$). This result shows that NFM has a greater potential for more immobilized and active β -galactosidase compared to PVDFM, GB, or NP.

The variation in apparent surface area, as seen in Figure 1, could relate to differences in enzyme binding potential. Increasing the surface area is presumed to increase the locations available for enzyme binding and would likely lead to greater observed enzyme activity.

Because there was no significant increase in mean enzyme activity for PVDFM, GB, or NP with increased enzyme concentration available during immobilization in this study, the maximum measured active enzyme binding capacity for these materials is likely to be at some concentration lower than 10 mg/mL.

When comparing the maximum enzyme immobilization capacity across the materials for each concentration, NFM had considerably greater means of enzyme activity than the other immobilized samples. At the lowest recorded concentration of enzyme in the immobilization solution, free and NFM were different from all other materials ($P \leq 0.01$ and 0.007 ,

respectively). For each material at 50 mg/mL, which was the point that none of the samples had a significant increase in mean activity with increased enzyme concentration in the immobilization solution, GB and NP were the only significantly different enzyme activity when compared with free ($P = 0.006$ and 0.001 , respectively) and NFM ($P = 0.008$ and 0.001 , respectively). Although NFM was not significantly different from PVDFM ($P = 0.177$), NFM was still 140% greater in mean activity. NFM and NP, although made from the same material, were the most significantly different in enzyme activity—NFM having 10 times more activity than NP at an available enzyme binding concentration of 50 mg/mL ($P < 0.001$). This suggests the increase in surface area due to electrospinning allows for more active enzyme bound, as expected.

Similar to the lowest concentration of immobilization solution enzyme, at 100 and 500 mg/mL free had significantly different activity than all other recorded materials ($P \leq 0.02$) besides NFM ($P \geq 0.98$). At 500 mg/mL immobilization solution enzyme concentration, NFM was also different from the other recorded materials PVDFM and NP ($P \leq 0.01$).

NFM and PVDFM were selected for further analysis because these materials showed the greatest ability for active enzyme attachment and had better feasibility for enzyme immobilization or implementation in dairy processing as compared to GB and NP.

The activity of β -galactosidase at an array of pH values was observed at 50 °C for β -galactosidase immobilized on NFM and PVDFM and were compared with free enzyme (See Figure 3).

Figure 3. Effect of pH on relative enzyme activity.

Highest mean activity for each material was normalized to 1 and the relative effect of pH on enzyme activity with each given material was compared. When comparing β -galactosidase activity across pH within the given materials, no difference was detected for NFM nor PVDFM ($P \geq 0.256$ and 0.144 , respectively). However, free enzyme exhibited significant variation in activity for most pH comparisons. Significant difference in activity is shown between NFM and PVDFM ($P = 0.029$) at pH 6.0. Error bars representing 1 standard deviation are shown. Free = free, dissolved enzyme, NFM = nylon-6 fiber membrane, PVDFM = polyvinylidene fluoride membrane. Letter codes denote statistical differences within material groups.

The greatest mean enzymatic activity for free enzyme was at pH 4.0, whereas both NFM and PVDFM had the greatest mean enzymatic activity at pH 5.0. The β -galactosidase activity remained consistent from pH 3.5 to pH 6.0 when immobilized on NFM ($P \geq 0.256$) and PVDFM ($P \geq 0.144$); however, variation in pH for the free enzyme resulted in significant statistical differences between most pH comparisons. Activity at pH 3.5 was different from all other pH conditions ($P \leq 0.008$) except pH 6.0 ($P = 0.918$) for free enzyme. Additionally, free enzyme activity at pH 4.0 showed significant differences from all other pH conditions ($P \leq 0.015$). Free

enzyme activity at pH 5.0 was not different at the neighboring pH values of 4.5 ($P = 0.152$), and 5.5 ($P = 0.138$); yet all other comparisons for pH 4.5, 5.0, and 6.0 were significant ($P \leq 0.002$).

Comparing activity results between the immobilized samples of NFM and PVDFM, before normalization of the data, the only significant difference ($P = 0.029$) shown by an unpaired, two-tailed t-test with Welch's correction is at pH 6.0; whereat NFM has a greater mean activity than PVDFM.

The immobilized enzyme compared to free enzyme has greater resistance to change in enzyme activity as the pH moves farther away from the optimal pH for β -galactosidase activity. The consistency in activity across pH conditions may very well be due to the enzyme being immobilized on a solid support material. The enzyme could be less likely to lose activity over more extreme pH conditions because of the tightly packed β -galactosidase acts as a localized buffer by modification of amino acid side chains that do not impact activity. The relative optimal pH for mean β -galactosidase activity shifted from 4.0 for free enzyme to 5.0 for the immobilized enzyme. Similar increasing shifts in optimal pH due to immobilization have been found in other work (Szczodrak, 1999). Fai et al., (2017) on the other hand, found optimum pH conditions for immobilized enzyme to be lower than that of free enzyme. Further research shows different directions of optimal pH shifting between immobilized and free β -galactosidase depending on the origin of the enzyme (Katriolia et al., 2018).

Although any given enzyme has an optimum pH for enzymatic activity, sometimes adjusting the substrate to the optimum pH of the enzyme is not an option. Other factors are at play, particularly within dairy processing, such as avoiding protein isoelectric points or pH differences due to cheese-type of the whey being treated (Nelson et al., 2004). These and other

limitations can result in a processing pH that is less than ideal for maximum β -galactosidase activity.

The variation in activity between samples, represented by error bars in Figure 3, do show less deviation from the mean for free enzyme samples as compared to immobilized enzyme samples. It is expected that more variation would occur replicating immobilized enzyme samples as compared to free enzyme samples because the immobilization process is not perfectly uniform.

In food processing, temperature of food products is also variable, like pH, and is monitored and regulated to reduce microbial growth, limit energy consumption, or enhance desired enzymatic reactions.

Figure 4. Effect of temperature on enzyme stability for immobilized enzyme on NFM, PVDFM, and free enzyme.

After initial enzyme activity was measured, samples were incubated in storage buffer solution at various temperatures. Activity was measured periodically over the period of 24 hours. Stability was defined as the time at which the relative activity reached 50%. The “>” symbol signifies that the sample retained more than 50% of the

activity by hour 24, and the percent of activity maintained at hour 24 is written above the corresponding column. Samples were performed in triplicate ($n = 3$). Free and PVDFM retained more activity at lower incubation temperatures. In contrast, NFM samples retained less activity, but maintained stability as temperature increased to 50 °C ($P \geq 0.094$). Free = free, dissolved enzyme, NFM = nylon-6 fiber membrane, PVDFM = polyvinylidene fluoride membrane. Letter codes denote statistical differences within material groups.

In order to know what affect altering the temperature had on the stability of the enzyme on various materials, three incubation temperatures were selected and the activity of the enzyme was measured periodically over the course of 24 hours of storage in the various temperature conditions. Because after 24 hours, free samples at 4 °C and 22 °C retained greater than 50% enzymatic activity as well as PVDFM at 4 °C, their relative activity at hour 24 is reported above their respective columns on Figure 4.

As incubation temperature was increased to 50 °C, mean enzyme stability decreased for both free ($P = 0.001$) and PVDFM ($P = 0.02$) samples. PVDFM started to decrease in mean enzyme stability after the increase from 4 °C and 22 °C ($P < 0.001$), unlike free ($P > 0.999$). NFM retained relative stability over the various temperatures ($P \geq 0.094$). However, NFM had a lower mean stability at 4 °C when compared with both free ($P = 0.014$) and PVDFM ($P = 0.012$). At 22 °C, NFM also had a significantly lower activity than free ($P = 0.014$) but not significantly lower than PVDFM ($P = 0.052$). At 50 °C, however, NFM did not have a different mean stability from free ($P = 0.605$), and had a higher mean stability than PVDFM ($P = 0.043$).

These results do not support the premise that immobilization of enzyme would increase mean stability as compared to free enzyme, as was found by Magro et al. (2019) and Misson et al. (2016). Some explanations as to why the results found in this study were different include the drop in enzymatic activity after each use (Hosseini et al., 2018; Katrolia et al., 2018)—a phenomenon limited to be witnessed only with immobilized enzyme. The free enzyme, although stored the same amount of time, cannot be reused once dissolved into a given solution, instead, a different sample is pulled (Katrolia et al., 2018). Lactase from *Penecillium notatum*

immobilized on alkylamine glass also lost activity over re-use by about 20% over 10 reuses of the immobilized enzyme (Szczodrak, 1999). Research by Mason et al. (2018) actually showed no significant difference in stability between free and immobilized invertase on NFM. Mohamad et al. (2014) suggest that the orientation of bound enzyme plays a critical role in determination of stability.

Figure 5. Effect of enzyme immobilization concentration on enzyme stability.

Free, NFM, and PVDFM materials with various immobilization solution enzyme concentrations were incubated in storage buffer pH 5.5 at 50 °C. Enzyme activity was read periodically, and stability was reported as the time at which relative activity reached 50%. Samples were performed in triplicate ($n = 3$). Statistical difference was found between free enzyme and the immobilized samples ($P < 0.001$). Whereas between NFM and PVDFM there was no such difference ($P \geq 0.119$). Free = free, dissolved enzyme, NFM = nylon-6 fiber membrane, PVDFM = polyvinylidene fluoride membrane. Letter codes denote statistical differences within material groups.

In order to determine if varying the β -galactosidase concentration in the immobilization solution affected stability, NFM and PVDFM samples—alongside free enzyme—were analyzed for stability by measuring enzyme activity over time (Figure 5). Just because there was no

difference in original activity for these concentrations of enzyme in immobilization solution, there may have been a difference in enzymatic stability. Within each material, varying immobilization solution enzyme concentration did not affect stability for NFM ($P \geq 0.779$) nor PVDFM ($P \geq 0.383$). These results support that varying immobilization solution β -galactosidase concentration between 50 and 500 mg/mL does not have a significant effect on immobilized enzyme stability.

When comparing the free, NFM, and PVDFM materials to each other across each concentration of enzyme in immobilization solution no significant difference was found ($P \geq 0.08$). From this study, it can be concluded that immobilizing β -galactosidase on these materials and under these conditions does not maintain nor enhance the stability of the enzyme as compared to free β -galactosidase.

Lowering the immobilization solution enzyme concentration sufficiently enough would decrease initial β -galactosidase activity. As discussed in Figure 2, maximum β -galactosidase activity was reached by 50 mg/mL β -galactosidase in enzyme immobilization solution for both NFM and PVDFM. It is for that reason that the concentration of 10 mg/mL was not tested for stability, because even with maintained stability, overall enzyme activity would not be optimal.

Figure 6. Effect of storage and reaction buffer pH on enzyme stability comparing free and immobilized enzyme.

Storage and reaction pH were adjusted to the various indicated values while temperature was maintained at 50°C during incubation with a standard enzyme immobilization concentration of 50 mg/mL. Activity was measured periodically over the course of 24 hours, and stability was determined as the time at which relative activity reached 50%. Samples were performed in triplicate ($n = 3$). Stability at pH 3.5 and 4.0 and all GB samples was considerably low and therefore not represented on this graph. At the higher pH conditions of 5.5 and 6.0, more variability in stability was seen between materials with free having a greater mean stability than the immobilized samples of PVDFM and NP ($P \leq 0.024$). Free = free, dissolved enzyme, NFM = nylon-6 fiber membrane, PVDFM = polyvinylidene fluoride membrane, NP = nylon-6 pellets, GB = silica glass beads. Letter codes denote statistical differences within material groups.

To observe the effect pH had on stability of free and immobilized β -galactosidase, free and immobilized enzyme samples were tested for stability in pH conditions of 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0. All samples at pH 3.5 and 4.0 lost essentially all detectable activity by the first incremental activity measurement, at hour 3, and therefore were not included in Figure 6. GB and NP were also measured for stability in various pH conditions. Potentially, GB or NP would have had a higher stability in a unique pH since the physical properties were quite different from the other materials; however, that was not found. GB lost all considerable activity before the first

stability reading was taken for every pH condition and therefore was not reported. All samples had greatest mean stability between pH 5.0 and pH 6.0.

Comparisons of pH storage conditions within each material were made. Free enzyme showed significant differences ($P \leq 0.014$) between all comparisons except between pH 5.5 and 6.0 ($P = 0.158$). NFM showed no significant differences between pH conditions ($P \geq 0.211$); again, likely because of the large standard deviation. PVDFM did show significant differences however, stability at pH 4.5 was different from both 5.5 ($P = 0.013$) and 6.0 ($P = 0.009$). NP showed significant difference in stability between pH 4.5 and both 5.0 ($P = 0.01$) and 5.5 ($P = 0.048$). The pH condition of 4.5 most often was significantly different most when compared to other pH conditions analyzed.

The effect of pH on enzyme stability was compared across the various materials also. At pH 4.5 and 5.0, no difference ($P \geq 0.059$ and 0.199 , respectively) was detected between all materials. At pH 5.5 and 6.0, free showed significant difference with PVDFM ($P = 0.024$ and 0.023) and NP ($P = 0.008$ and 0.013). PVDFM and NP were also different at pH 5.5 and 6.0 ($P = 0.012$ and 0.018). NFM showed no significant differences ($P \geq 0.065$) with the other samples at all pH conditions which is likely due to a greater standard deviation. In that light, the mean stabilities of NFM samples under all pH conditions measured were higher than the corresponding mean stabilities of PVDFM samples—supporting the possibility that NFM could provide better stability than PVDFM over varying pH conditions.

The variation for stability results is generally greater than the variation for activity results (compare Figures 3 and 6 and figures 5 and 1) thereby limiting the significant differences between samples. This may be due to the fact that activity is lost relatively exponentially over time so it is easy for the hour at which 50% of the initial activity is lost to shift dramatically

across the x-axis of time resulting in greater variation of the reported hour in connection with stability.

Comparing Figures 3 and 6 demonstrate that having greater initial activity does not mean that same pH condition will provide for greater stability as seen at pH 4.0 for free enzyme. It should also be noted that the comparison of the stability of β -galactosidase in any given condition does not account for the initial activity. Therefore, determining the ideal pH should not be made without considering both activity and stability if prolonged enzyme use is desired.

CONCLUSIONS

NFM showed a greater capacity to bind β -galactosidase than other analyzed support materials. NFM was also more resistant to loss of enzyme stability as temperature increased than free and PVDFM samples. At lower temperatures, however, NFM is not as enzymatically stable as other samples. Benefits seen from NFM are believed to be in connection with the larger surface area due to the micro-level structure of the membrane made up of fibers developed through electrospinning. Only membrane-immobilized β -galactosidase samples had no significant variation in enzyme activity over varying pH conditions. However, free enzyme showed greater stability relative to the immobilized enzyme at optimal pH—which is somewhat unique to this study. The stability of the immobilized enzyme might be increased with further modification such as PEGylation (Moreno-Pérez et al., 2016) or pre-treatment with lactase (Song et al., 2010), which would be worth exploring in future research.

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APPENDIX A

Full Review of Literature

Lactose Intolerance

About 70% of the world adult population has some form of lactose intolerance (Elnashar and Hassan, 2014). However, this sugar is very abundant and commonly found in food products. It is the most commonly consumed carbohydrate by infants due to its natural presence in human breast milk (Adam et al., 2004). Lactose is also commonly consumed by adults in the form of cow's milk or dairy ingredients. Milk and dairy products contain usually 5-10% (w/v) lactose (Elnashar and Hassan, 2014). However, for those who experience lactose intolerance, many products must be limited or even avoided. Others choose to suffer the discomfort that comes with the consumption of lactose if they have lactose intolerance. Lactose intolerance typically comes from a loss of activity of the lactase enzyme (Adam et al., 2004). The lactase enzyme is important for lactose metabolism because lactose, chemically defined as O- β -D-galactopyranosyl-(1-4)- β -D-glucose, is a disaccharide carbohydrate and must first be broken down into its monosaccharide components—glucose and galactose—in order to be absorbed in the small intestine (Adam et al., 2004). Due to damage of the intestines, other conditions can lead to temporary lactose intolerance such as Leśniweski-Crohn disease, ulcerative colitis, and after chemotherapy or intestine surgery (Strzałkowska et al., 2018).

Reduce Lactose Through Fermentation

Mammalian milk typically contains lactose which can be reduced via fermentation (Strzałkowska et al., 2018). Fermentation is the metabolic breakdown of carbohydrates into carbon dioxide, lactic acid, alcohols, and other end products (Pasotti et al., 2017). Lactic acid bacteria are a common group of bacteria which produce mainly lactic acid as an end product of carbohydrate fermentation. Lactic acid bacteria are commonly used in dairy foods production

(Adam et al., 2004). For example, Lactic acid bacteria are utilized in the production of yogurt, kefir, cheese, buttermilk, and sour cream. Some yeast also has the capacity to ferment lactose into other various end products including the genus *Kluyveromyces* (Adam et al., 2004). Other beneficial side effects of fermentation include a decrease in food pH and an increase of bacterial competition. These factors help to maintain food safety and avoid pathogenic bacterial growth.

There are other methods of decreasing lactose content, one of which is enzymatic hydrolysis of lactose. Lactase enzymes are designed to accomplish a very similar job as lactic acid bacteria, or other lactose-fermenting organisms. Some food products inherently utilize fermentation for their production whereas others are made uniquely lactose-free or reduced in lactose for some other purpose—commonly to allow individuals with lactose intolerance to consume the food. In a study performed by Skryplonek et al. (2017), frozen yogurt was treated with lactase to reduce lactose content. Sensory and analytical tests were performed to note any differences. Although fat, protein, and ash content remained practically the same, sweetness increased and the textural and melting properties improved due to the alterations of the concentrations of glucose and galactose after hydrolysis. The fermentation of lactose additionally reduced the likelihood of sandiness defect in frozen dairy products because the grittiness is due to α -lactose crystals (Skryplonek et al., 2017). Although changes to the food occur due to lactose hydrolysis, some changes may be desirable or enhance the product.

Galactooligosaccharides

Galactooligosaccharides are one desirable end product produced during the hydrolysis of lactose. Galactooligosaccharides are a pre-biotic and are typically used in nutraceuticals (Skryplonek et al., 2017). They have potential benefits including increasing milk digestibility, increasing absorptions of different minerals in the intestine, reducing toxic metabolites, and

depressing blood pressure (Fai et al., 2017). Galactooligosaccharides are formed by the enzymatic action of beta-galactosidase which transfers galactose onto either a water molecule—which forms the monosaccharide galactose—or onto another galactose molecule—which forms or elongates a chain. The process and reaction time of lactose hydrolysis can be altered in attempts to produce a relatively greater concentration of galactooligosaccharides (Fai et al., 2017).

Lactose Labeling and Added Sugars

In May of 2016 a revision to the nutrition and supplement facts labels was announced. By January 2020 or 2021 (depending on manufacturer size), food manufacturing companies will need to have updated their nutrition facts panel and serving size. One of the major changes to the nutrition facts label is the listing of “added sugars.” Lactose in milk and milk products has been clearly defined as to not count towards “added sugars,” but rather, “total sugars” only. However, there have been questions of labeling the sugars produced through enzymatic hydrolysis of dairy products. If the hydrolyzed lactose product meets the definition of “lactose” as described in 21 CFR 168.122 then the product would be labeled as “added sugars” (U.S. Department of Health and Human Services Food and Drug Administration Center for Food Safety and Applied Nutrition, 2018). Because one of the requirements to meet the definition of “lactose” is that the content is not less than 98.0 percent (m:m) calculated on a dry basis, then dairy sugar products with less than 98% lactose purity would not be defined as “added sugars” (21 CFR 168.122 (d) (1)). Unpurified whey permeate has a lactose content of 76-85 percent, with 3-5 percent moisture; therefore, whey permeate would not be declared as an added sugar (American Dairy Products Institute, 2020). With the application of hydrolyzing or isomerizing enzymes to whey permeate, the perceived sweetness would increase without the need to declare an increase of

“added sugars” (Illanes et al., 2002). Utilizing hydrolyzed lactose in dairy products may very well be a main option for food manufactures to cut back on declared added sugars on their products’ nutrition facts labels and maintaining targeted sweetness levels. This solution could have a significant positive impact for the dairy industry.

Enzymes—Lowering Activation Energy

Enzymes are catalysts that lower the activation energy, or energy required to initiate a chemical reaction. Without enzymes, many reactions would take so long that they would not noticeably occur. Enzymes convert substrates into products without permanently being altered themselves (Mohamad et al., 2015). The way enzymes convert substrate into product is by forming a substrate-enzyme complex which then puts the molecules in position for the specific reaction to occur (Eq. 1).

Even though enzymes are so useful, they also have limitations. Product concentration, temperature, pH, ionic strength, and substrate availability are all conditions which affect enzymes’ capacity to aid in the reaction process. Under optimal conditions, enzymes can function at their fastest rate and maintain the structural integrity required for activity. The optimal rate and the maintenance of structural integrity is defined by the enzyme’s activity and stability.

Enzyme Activity and Stability

The activity of an enzyme is the rate at which the enzyme converts substrate into product. As an enzyme helps to perform a chemical reaction, heat is either released or absorbed. The heat is directly related to the reaction, and the reaction refers back to the definition of enzyme activity

(Rohatgi et al., 2015). As the change in rate of heat increases or decreases and is measured, the activity of the enzyme can be calculated (Mason et al., 2018).

If an enzyme's environment is optimal, then the enzyme can work more efficiently and convert substrate into product faster (Salleh, 1982). As an enzyme is reused over and over, or if it is in a sub-optimal environment the enzyme loses activity until it is no longer able to perform its proper function. The ability for an enzyme to retain activity over time and over various environmental conditions is the enzyme's stability (Jin et al., 2017).

Immobilized Glucose Isomerase and Corn Syrup

Understanding enzyme functionality helps to better utilize enzymes and optimize processing involving enzymes. Enzymes are commonly applied in the food industry and enzyme immobilization has been utilized for at least a half a century (Strandberg and Smiley, 1972). One very common example is the use of immobilized glucose isomerase in the production of high-fructose corn syrup. Immobilizing the enzyme entails covalently attaching the enzymes to porous beads and then placing them into a column where the sugary solution passes through the beads. During contact with the immobilized glucose isomerase beads, the glucose in solution is converted to fructose. This fructose is used as a primary constituent in high fructose corn syrup (HFCS). HFCS is used in food products as a cheaper alternative to replace beet or cane sugar while maintaining or increasing relative sweetness (Jin et al., 2017).

Other enzymes utilize the same immobilization technique in order to increase processing efficiencies and produce food products that are economical (Szczo drak, 1999). Although the immobilization of glucose isomerase has been studied for many years, other enzymes have not been applied in processing for as long (Chen et al., 1981). Therefore, more research could be

done to understand the characteristics of other enzymes and improve processing conditions for enzyme functionality optimization.

Free Enzyme

In contrast to immobilized enzymes, there are examples of free (also known as soluble or dissolved) enzymes utilized in the food industry. Currently, in order to make lactose-free milk, the bath-method is used where enzyme is added to the milk, allowed to react and hydrolyze the lactose, and then heating the mixture to denature the enzyme. The product can then proceed to packaging (Zhao et al., 2017). Another example of the utilization of free enzyme is invertase. Invertase catalyzes the hydrolysis of sucrose which results in what is known as invert sugar. Invert sugar is sweeter than sucrose due to its monosaccharide components, glucose and fructose. This extra-sweet sugar is used primarily in the beverage industry (Amaya-Delgado et al., 2006).

Enzyme Immobilization

The use of enzymes is expanding past food and into biocatalysts, biosensors, and medicines. Immobilization is one way to increase efficiency of enzyme-catalyzed bioprocesses which are becoming more popular due to the fact that enzyme use is “green” and sustainable in comparison with chemical processes. By immobilizing enzymes, enzymatic processing becomes more affordable due to an increase in stability and reusability. Batch processing may also be converted into continual processing which is beneficial for production efficiency.

The two main types of enzyme immobilization are chemical and physical. Chemical attachment methods are stronger and have multiple bonds formed; therefore, chemical attachments are preferred. The four major kinds of chemical enzyme immobilization are adsorption, entrapment, covalent, and cross-linking (Mohamad et al., 2015). Adsorption and covalent coupling are two of the most common methods; however, adsorption can lead to

leaching and covalent coupling can be harsh and lead to an undesirable loss of enzymatic activity (Fai et al., 2017). Ultimately, an attachment method is chosen to complement the needs of the process and avoid risks of enzyme or stable support pieces becoming detached during processing.

In the process of covalent immobilization, a stable support is selected and prepared for enzyme immobilization. In order to increase enzyme binding, modifications are made to the stable support before being introduced into the enzyme solution (Misson et al., 2016). Particularly, many carboxylic acid, epoxy, and hydroxyl groups act as anchoring sites for the protein (Misson et al., 2016). In the process of immobilizing beta-galactosidase, the stable surface is commonly activated with glutaraldehyde in order for the enzyme to attach (Szczodrak, 1999). The addition of glutaraldehyde also assists with retaining enzymatic activity. In a study done on beta-galactosidase immobilization onto celite, 27% activity was retained when glutaraldehyde was used, as compared to 11.3% activity retention in the absence of glutaraldehyde (Fai et al., 2017). Additionally, glutaraldehyde has been used in the preparation of other immobilized enzymes, such as glucose isomerase (Suekane, 1982). Genipin has been explored as an alternative to glutaraldehyde—claiming benefits of having a much lower toxicity level and being a naturally occurring bi-functional crosslinking reagent (Klein et al., 2016).

Stable Supports

A variety of stable supports have been used for enzyme immobilization. The stable support acts as the solid surface onto which enzymes are immobilized. Stable supports can come in many forms including beads, membranes, microbeads, and resins. The range of materials in which the stable support is made from is even broader. The most common stable supports include: carboxymethyl-cellulose, starch (Misson et al., 2016), collagen, modified sepharose, ion

exchange resins (Suekane, 1982), active charcoal, silica (Gennari et al., 2018), clay, aluminum oxide, titanium, diatomaceous earth (Wahba, 2017), hydroxyapatite, ceramic, celite, agarose (Mateo et al., 2004), treated porous glass which is an organic material (Szczodrak, 1999), and certain polymers (Mohamad et al., 2015).

Knowing which factors to consider is helpful in selecting an appropriate stable support. Cost, availability, functionality, hydrophilicity, inertness towards enzymes, biocompatibility, biodegradability, resistance to microbial attack, resistance to compression, shape, size, toxicity, and ease to produce are all aspects that may need to be considered before selecting a stable support. There is no one ideal support for every immobilization procedure (Mohamad et al., 2015).

Binding Capacity of Stable Support

One key factor in determining the material for the stable support is its ability to bind enzyme. The surface-to-volume ratio of the material is generally referenced in order to determine binding capacity (Rodríguez et al., 2018). Electrospinning is a method that is used to create high surface-to-volume ratio nanofiber membranes. A polymer, such as nylon-6 has been dissolved in formic acid and used to form nanofiber strands that create a network available for enzyme immobilization (Mason et al., 2016).

The addition of a spacer molecule further increases the binding capacity of the stable support (Salleh, 1982). Adding PEI, for example, can increase adsorption points for the polymer and increase overall stability (Moreno-Pérez et al., 2016).

Effect of Enzyme Immobilization

Changes in optimum temperature and pH are common after enzyme immobilization. These changes could be due to the chemical modification of the enzyme and from the physical

interference by the stable support. The kinetic behavior differences due to the physical interferences could arise from electrostatic and steric influences or the time required for the diffusion of substrates and products (Szczodrak, 1999).

Activity

Even though there has been much work done on optimizing immobilized enzymes, it is common that enzymes lose some activity due to the immobilization process (Misson et al., 2016). In one study, the activity of β -galactosidase decreased from 11.98 U/mg to 3.23 U/mg after immobilization (Fai et al., 2017). It is supposed that the random orientation of the enzyme during immobilizing may lead to the inaccessibility of some of the enzyme's active site (Mason et al., 2016).

Stability

Typically, the stability of the immobilized enzyme is much greater than that of free enzyme (Strandberg and Smiley, 1972). Commonly, immobilization of the enzyme increases the temperature of instability of the enzyme by approximately 10 °C (Szczodrak, 1999). For example, the half-life of immobilized glucose isomerase was 12-14 days instead of 1 day for free enzyme, or batch operation (Strandberg and Smiley, 1972). A different study found the half-life of that same glucose isomerase enzyme to be 1,000 h, or over 41 days (Chen et al., 1981). Another example of increased stability of immobilized enzymes shows invertase had a T_{max} of about 67.3 °C when free, and 68.8 °C when immobilized (Mason et al., 2018). Immobilized beta-galactosidase was reused for 10 cycles while retaining at least 86% of its activity, showing the ability for reuse of the immobilized enzyme. Beta-galactosidase also showed a 30% reduction in activity at 60 °C when free, compared to only a 15% reduction in activity when immobilized (Szczodrak, 1999). Additionally, after 270 days of storage, suspended in 0.1 M acetate buffer,

pH 4.5 at 3.5 °C, the enzyme retained 85% of its residual enzymatic activity (Fai et al., 2017).

This data suggests that immobilized enzyme may be stored for months without a significant drop in enzymatic activity.

Enzyme Concentration

Generally, an increase in enzyme would increase the overall reaction rate of converting substrate into product; although a limit can be reached where an increase in enzyme concentration does not increase reaction rate. Results showed that increasing the concentration of beta-galactosidase from 10 U to 60 U showed almost no increase in enzyme loading capacity after the maximum of 36 U/g stable support was reached (Elnashar and Hassan, 2014). In other research, relative activity decreased once enzyme loading exceeded 138 U/g of chitosan support (Rejikumar and Devi, 2001). The substrate concentration (lactose) was 200 mM (Elnashar and Hassan, 2014).

Temperature

The optimal temperature typically increases by 5-10 °C after the process of immobilization (Gennari et al., 2018). For beta-galactosidase, immobilization increased optimal temperature from the range of 30-37 °C to 37-40 °C (Elnashar & Hassan, 2014). Similarly, invertase optimal temperature increased from the range of 55-65 °C for free invertase to 65-70 °C for immobilized invertase (Amaya-Delgado et al., 2006). Optimal temperature may fluctuate depending on flow rate of the substrate. A slower flow rate is less temperature-dependent whereas a faster rate favors higher temperatures (60 °C as opposed to 50 or 40 °C) for immobilized enzyme (Rejikumar and Devi, 2001). The shift in increasing optimum temperature indicates the structure of the enzyme is strengthened by the immobilization process—forming a

molecular cage around the enzyme molecule which results in increased stability (Elnashar and Hassan, 2014).

Temperature also affects enzyme stability. If the temperature of the environment of the enzyme is too warm, the result is denaturation of the enzyme and therefore loss of activity. It was found that immobilization of beta-galactosidase helped to maintain activity better at higher temperatures as compared to free enzyme (Rejikumar and Devi, 2001). Stability of beta-galactosidase increased from 45 °C (free enzyme) to 55 °C (immobilized enzyme) in one report (Szczodrak, 1999). In another report, stability of beta-galactosidase increased from 40 °C (free enzyme) to 50 °C (immobilized enzyme) (Fai et al., 2017).

pH

Similar to temperature, pH is an indicating factor of how well an enzyme can function. For every enzyme, there is a particular optimum pH or pH range that will lead to better activity. However, there are variances in the literature of what that optimum is, depending on other conditions and procedures; although, there is a trend for free versus immobilized enzyme. Immobilized glucose isomerase was able to function over a wider range of pHs as compared to free enzyme; although the optimum remained about 7.3 when either free or immobilized (Chen et al., 1981). For beta-galactosidase, one study indicated the optimum pH remained constant over various temperatures for immobilized enzyme (Rejikumar and Devi, 2001). Other research reported a lowering of optimal pH after immobilization: the pH optimum was 4.6 for free enzyme and 4.0 for immobilized enzyme—which is a different trend than many other reported optimum pH shifts for enzyme immobilization (Fai et al., 2017). Other experiments show a shift in optimal pH range from 4.5-5.0 to 4.0-6.0 before and after immobilization respectively (Elnashar and Hassan, 2014). Along with that, the highest pH at which dissolved lactase could

perform was pH 4.0. Contrastingly, immobilized lactase was stable between pH 4.0-5.0—favoring 5.0 (Szczołrak, 1999). The reason for changes in pH optimums is speculated to be due to partition effects that may be arising from different concentrations of charged species in the microenvironment of the immobilized enzyme and in the domain of the bulk solution (Elnashar and Hassan, 2014).

PEGylation

In efforts to improve enzyme stability, enzymes may be surface-coated with polyethylene glycol (PEG). Physicochemically adding this dense, viscous, polar PEG layer around the enzyme, while still permitting access to the active site of the enzyme, is known as PEGylation. The extra protective layer helps stabilize the enzyme in harsh conditions including organic solvents, extreme pHs, high ionic strengths, or digestion by macrophages, and high temperatures. With the extra coating, the enzyme maintains activity better and can function under a wider range of environmental conditions (Moreno-Pérez et al., 2016).

The PEGylation connection to the enzyme is formed by an aldehyde-dextran polymer linkage. First, the dextran polymer is attached onto the enzyme surface that has been enriched with primary amino groups and then the aldehyde end is available for attachment with the PEG. Because the PEG polymer is hydrophilic, the hydrophobic interior of the enzyme, which typically contains the active site, remains uncoated. In order to enrich the enzyme surface with primary amino groups, ionic adsorption of polyethyleneimine (PEI) or polyallylamine (PAA) on the ionized carboxyl groups of the enzyme (Asp + Glu residues) is utilized. With the extra amination, the stability of the enzyme, glucose oxidase, has shown a 50-fold increase in stabilization at 40 °C (Moreno-Pérez et al., 2016).

Measuring Enzyme Activity Spectrophotometrically

Without the use of calorimetry, the methods for measuring enzymatic properties are limited to various spectrophotometric methods. Typically, in order to measure enzyme kinetics, activity, and stability, a coupling or tagging of substrates is needed in order to produce a color change that can be measured and related back to the reaction (Rohatgi et al., 2015).

o-nitrophenyl- β -D-galactopyranoside (ONPG)

Activity of beta-galactosidase has been measured using o-nitrophenyl- β -D-galactopyranoside (ONPG) as the substrate (Fai et al., 2017). ONPG undergoes hydrolysis which liberates ONP (dos Santos et al., 2017). This hydrolysis reaction induces a color change from transparent to yellow (Labus, 2018). The enzyme activity is then related to the amount of enzyme that liberated 1 μ mol of o-nitrophenol (ONP) per minute under the standard assay conditions using Beer's law (Fai et al., 2017; Rodríguez et al., 2018). Measurements of the yellow color generated are taken using an absorbance measurement at 405 nm on a spectrophotometer (Mateo et al., 2004).

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APPENDIX B

Effect of immobilization solution enzyme concentration, Free stability

Effect of immobilization solution enzyme concentration, NFM stability

Effect of immobilization solution enzyme concentration, PVDFM stability

Effect of storage and reaction buffer pH on enzyme stability Free

Effect of storage and reaction buffer pH on enzyme stability NFM

Effect of storage and reaction buffer pH on enzyme stability PVDFM

Effect of storage and reaction buffer pH on enzyme stability GB

Effect of storage and reaction buffer pH on enzyme stability NP

Effect of temperature on Free, normalized stability

Effect of temperature on NFM, normalized stability

Effect of temperature on PVDFM, normalized stability

APPENDIX C

The variation for stability results is generally greater than the variation for activity results thereby limiting the significant differences between samples. This may be due to the fact that activity is lost relatively exponentially over time so it is easy for the hour at which 50% of the initial activity is lost to shift dramatically across the x-axis of time resulting in greater variation of the reported hour in connection with stability. A deeper explanation is shown below.

Absorbance was measured over time (s) and then converted to mmol ONP produced/L using Beer's law and plotted as an activity curve. The linear regression slope of each activity curve replicate was plotted for each measured hour on the stability graph.

The point at which each stability curve replicate reached 0.5 was recorded and the average was graphed along with the standard deviations.

APPENDIX D

Instructions for Electrospinning

Start off with putting on the appropriate PPE (gloves, goggles, lab coat)

The electrical equipment which produces the electrical current will need to be grounded.

We have it grounded to the circuit considering our location.

Align the back of the electric receiver to about 11.5 cm so the front of the receiver will be at 11 cm. I have a piece of paper slid in between the cardboard and the aluminum foil in order to try to flatten out the surface of the receiving board. I additionally have a piece of wax paper taped to the front of the aluminum foil for the membrane to collect on and then easily be removed later.

To load the syringe with dissolved nylon-6, remove the plunger from a 5 mL plastic syringe and carefully pour the nylon-6/formic acid solution into the syringe from the back filling

it up 2-5 mL full while plugging the tip with the packaging paper. Carefully insert the plunger just enough to form a seal and then invert the solution-filled syringe so the tip is upward. Allow air bubbles to rise to the top and then push in the plunger until there is no significant air bubble. Then screw on the stainless-steel needle and push the solution with the plunger until a small amount comes out the tip. Place the loaded plunger into the automatic syringe pump set to 0.001 - 0.002 mL/min.

Attach the two electrical ends onto the needle and onto the collection plate.

The pump should be on and the tip of the needle should be aligned at the 0 cm mark.

At this point the electrical source can be turned on. The current should be very low (about 1 to 2 ohms) and the voltage should be high (about 25 to 30 kV). The needle measuring voltage needs to be steady. Starting from a higher current and slowly moving to a lower current can be useful to help maintain voltage.

The membrane should be noticeable after an hour and can take between 3-8 hours to form a membrane. The longer the pump is running, the thicker the membrane will be.

Once the membrane is completed, turn off the electrical power source and unplug it. Then you can turn off the pump and remove the wax paper. Carefully peel off the membrane by carefully rolling the edge of the membrane until you can lift the membrane from the wax paper. Take care while handling the membranes. The structure can be altered with pinching, pressing, cutting, etc. The membranes can be stored in a dry place at ambient temperature.