**Development of Magnetic Nanobiocatalyst for Economic Biorefinery of Soybean Products**

**Abstract**

Development of soybean alternative products is a potential way to increase the use of soybean locally and internationally. However, many biochemical reactions use smaller molecules (oligomers and monomers) than the soybean polymers (proteins and starch). The breaking down of soybean protein and starch is done using enzyme (biocatalyst). Therefore, the aim of this research is to develop magnetic enzymes for breaking soybean product (dehulled, fatted flakes, defatted flakes, and soymeal) protein and starch. Magnetic and non-magnetic nanobiocatalysts containing both amyloglucosidase (AMG) and amylase (AMY) were developed to breakdown starch while nanobiocatalyst of pepsin were developed to breakdown protein. The nanobiocatalyst performances varied among the soybean products because of the compositional difference. Simultaneous addition of AMG and AMY performed higher than separate addition therefore nanobiocatalyst containing both AMG and AMY were developed. Non-magnetic nanobiocatalyst performed higher than magnetic nanobiocatalyst. Pepsin attached to polymer performed lower than free pepsin, in all samples except dehulled soybeans. Attaching enzyme to flexible polymer is potential cost reduction approach for soybean hydrolysis in biorefinery operation.

**Keywords**: immobilization, enzyme hydrolysis, magnetic nanobiocatalyst, soybean

**Introduction**

Soybeans contain an array of biomolecules that potentially could be used for food, feed, health, and chemical synthesis applications. Developing soybean alternative products could ameliorate the rising tensions and new export barriers that have disrupted US soybean exports and have affected soybean growers economically. However, some alternative applications are not yet possible because of the high processing temperature used in conventional soybean processing. Conventional soybean processing denatures soybean biomolecules thereby becoming less fit as precursors for above mentioned applications.

Enzyme-based processing has been identified as an alternative to chemical processing because of the low operating temperature. Enzymes are also more easily controlled thereby preventing side reactions and allowing the use of water or reducing solvent requirement. However, the high enzyme cost makes this prohibitive to industrial processes. An enzyme-based process could become economical if enzymes are recycled. Enzyme recycling is possible if they are immobilized on supports that ease recovery and reuse.

We aim to attach different enzyme types on magnetic nanoparticles to catalyze soybean bioprocessing. Specifically, we developed and tested magnetic and non-magnetic polymer enzyme conjugate containing amylase, amyloglucosidase and pepsin for hydrolysis of starch and protein from four soybean products (dehulled, fatted flake, defatted flake, and soymeal) that are commonly found in soybean processing plant.

**Methods**

Sample preparation

Four soybean samples (dehulled, fatted flake, defatted flake and soy meal) were collected from soybean process plant located in Fargo, North Dakota. The sample were grinded using electric blender and stored at 4oC until needed.

Proximate Composition analysis

The protein, fat, ash, and moisture carbohydrate contents of each sample were determined, respectively, using total Nitrogen method, Soxhlet method, dry ashing, oven drying method and subtraction method.

Magnetic nanobiocatalyst synthesis

The polymer ligand (Poly (GMA-*co*-PEGMA)) for enzyme immobilization was a copolymer of glycidyl methacrylate (GMA), and poly (ethylene glycol) methyl ether methacrylate (PEGMA300 –average molecular weight 300 g/mol). The copolymer was dropwisely added to enzyme solution and mixed overnight to produce polymer enzyme conjugates (PECs). The PECs adsorbed on superparamagnetic nanoparticles to make magnetic nanobiocatalyst.

Sample hydrolysis

A mass of 0.5 g of each sample were weighed into 50 ml conical flask and 10 ml of buffer containing enzymes were transferred. The hydrolysis was conducted in water bath under agitation at 130 rpm. For starch hydrolysis 0.02 g/ml of amylase (AMY) and amyloglucosidase (AMG) were used at pH of 5.3 and 70oC while for protein hydrolysis 0.002 g/ml of pepsin was used at pH 2, and 35oC.

Hydrolysate analysis

Glucose content of the hydrolysates were determined using HPLC and glucose as standard. The amino acid contents of the hydrolysates were determined using TNBS method and glycine as standard.

**Results**

***Proximate composition of soybean products***

To develop immobilized enzymatic process that is adoptable by soybean processing industries, four different soybean streams including dehulled, flaked, defatted flaked and meal of soybeans identified. The result of proximate composition of the soybean streams are presented in Table 1. The result showed that the proximate composition varies among the sample streams. The moisture content was lowest in oil flake and highest in soymeal. All samples showed high protein and carbohydrate contents with values, respectively ranging from 35 – 41 % and 37 – 44 %. All samples streams are low in ash content. The oil content was high in oil flake followed by dehulled soybean, then soymeal and then defatted flake.

The variation and structure composition of the samples affect the enzyme process efficiency. Presence of high amount of protein and carbohydrate suggest the need for protease and carbohydrase for process development. Hence, the proposed nanobiocatalyst must contain both enzymes. While pepsin will be suitable to breakdown all forms of protein, there is no single carbohydrase that can breakdown all carbohydrate. Soybean carbohydrate mostly contain starch. Therefore, the carbohydrase that will be needed include amylase and amyloglucosidase.

Table 1: Proximate composition of dehulled soybeans, oil flake, defatted flake and soy meal

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Sample | Moisture Content (%) | Protein (%) | Oil  (%) | Ash  (%) | Carbohydrate (%) |
| Dehulled soybean | 8.06±0.04 | 34.68±0.69 | 15.31±0.38 | 5.43±0.00 | 36.52±0.23 |
| Oil Flake | 6.85±0.22 | 33.36±1.12 | 21.32±0.05 | 5.30±0.12 | 33.17±0.15 |
| Defatted flake | 7.82±0.79 | 41.03±1.20 | 0.98±0.01 | 6.63±0.03 | 43.54±0.54 |
| Soymeal | 11.31±0.05 | 39.31±4.60 | 1.32±0.01 | 6.84±0.02 | 41.22±0.40 |

***Effect of amylase and amyloglucosidase addition mode on soybean product hydrolysis***

Starch structure may be altered through various enzymatic modifications to achieve desired properties that is normally not inherent (Jayakody, L., & Hoover, R. 2002). For starch hydrolysis to proceed, it requires two enzymes – amylase (AMY) and amyloglucosidase (AMG) (A. Dura et al., 2014). As earlier demonstrated by previous researchers, addition of AMY and AMG was carried out using 2 major approaches series and parallel. The series approach involves the stepwise use of amylase and AMG (one after the other) while parallel approach involves the application of both enzymes simultaneously. Miao et al., (2011) and Han, X. Z., et al., (2006) utilized the parallel mode for waxy corn starch hydrolysis while A. Dura et al., (2014) used series mode.

The four samples were subjected to hydrolysis using the AMY and AMG in series and in parallel. Figure 1 shows that the soybean samples were hydrolysis differently. These differences could be attributed to their varying structural composition, molecular characteristics, crystalline organization or even their starch content. Parallel addition of AMY and AMG was higher than series in all samples, except in defatted flakes where both parallel and series have similar result. The highest hydrolysis was achieved at 3h, except for dehulled sample. Subsequent hydrolysis starch experiments were therefore carried for 3 h and parallel addition of AMY and AMG.

B

A

D

C

Figure 1: Glucose contents of soybean hydrolysates using parallel and series addition of amylase and amylogulcosidase. A: dehulled soybeans, B: oil flakes, C: defatted flakes, and D: soy meal.

***Effect of polymer conjugation of amylase and amyloglucoside on soybean product hydrolysis***

Prior to producing magnetic nanobiocatalyst, a non-magnetic nanobiocatalysts of AMY and AMG were synthesized by attaching the enzymes to flexible polymer. Figure 2 shows that the non-magnetic nanobiocatalyst improved soybean product hydrolysis compared to free enzymes in Figure 1. Attaching enzymes to flexible polymer create enzyme colony that works on substrate synergistically. This possibly explains the reason for higher performance compared to free enzymes. Figure 2 also shows that attaching both AMY and AMG on flexible polymer is preferred than having just one of the enzymes attached.

Figure 2: Glucose contents of soybean product hydrolysates using combination of polymer enzyme conjugates (PECs) and free enzymes (FE) at different cases: case 1: AMG-PEC and AMY-PEC, case 2: AMG-PEC and FE-AMY, and case 3: AMY-PEC and FE-AMG

***Effect of amylase and amyloglucoside attachment on magnetic nanoparticle on soybean product hydrolysis***

To produce magnetic nanobiocatalyst for starch hydrolysis, AMY-AMG-PECs were attached on magnetic nanoparticles and used for hydrolysis of soybean products. Figure 3 showed that the magnetic nanobiocatalyst performed differently among the samples. However, magnetic nanobiocatalyst of AMY and AMG performed lower than free enzyme. This could be due to reduction in accessibility to substrate by magnetic nanobiocatalyst.

Figure 3: Glucose release during soybean product hydrolysis using magnetic nanobiocatalyst

***Protease hydrolysis of soybean products***

To determine the effect of polymer enzyme conjugation in pepsin efficacy, four samples of soybeans (dehulled, fat flake, defatted flake and meal) were hydrolyzed using polymer enzyme conjugate of pepsin (Pepsin-PEC), magnetic nanobiocatalyst of pepsin (Pepsin-MNBCs) and free pepsin (Pepsin-FE). Figure 4 shows that after 6h of hydrolysis, the amino acid (glycine equivalent) of the hydrolysates were measured using TNBS method. Compared to Pepsin-PEC, Pepsin-FE have higher amino acid contents in all samples except in dehulled sample. This observation shows that the effect of polymer enzyme conjugation on pepsin efficacy is sample dependents. Presence of polymer could affect the interaction of enzyme with substrate. The difference in sample composition can cause difference in polymer substrate interaction which in turn affect enzyme efficacy. Since the oil in fatted flake are easily accessible, the polymer might have interacted more with oil than protein suggesting the reason for high difference in pepsin efficacy in fatted flake compared to defatted flake and soy meal.

Figure 4: Amino acid content of soybean product hydrolysates using polymer enzyme conjugate of pepsin (Pepsin-PEC) and free pepsin (Pepsin-FE) for 6 h at 35 oC.

**Conclusion**

Soybean contain more starch and protein and requires multiple enzymes for complete hydrolysis to smaller molecules for bioprocessing. The high cost of enzymes is among the factors prohibiting biorefinery processing of soybeans. Enzyme immobilization can make enzyme recovery and reuse possible. Attaching enzymes on flexible polymer that was then attached on magnetic nanoparticles is a potential means to make enzyme recovery and reuse possible. The mode of application of AMY and AMG affects hydrolysis. The hydrolysis varies among samples. Unlike pepsin, attaching AMG and AMY on flexible polymer did not affect hydrolysis of most soybean products. Future studies should include fermentation of hydrolysates to organic ammonia.

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