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MPSG ANNUAL EXTENSION REPORT

PROJECT TITLE: Joining the Green Revolution: Value Added Fermentations of Peas and Beans

PROJECT START DATE: May 1, 2014
PROJECT END DATE: April 30, 2016
DATE SUBMITTED: January 27, 2015

PART 1: PRINCIPAL RESEARCHER

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PART 2: EXECUTIVE SUMMARY

This project will investigate the use of pea and bean meal as an inexpensive nitrogen source in typical commercial fermentations such antibiotic production and lactic acid production (polylactide plastics) and as a fermentation promoting ingredient in fuel and beverage ethanol fermentations. Fermentations will be performed using standard media formulations and then the conventional nitrogen source will be replaced with pea or bean meal and the effect of the substitution on the particular fermentation will be measured. We will also hydrolyze commercially available pea protein with commercially available protease enzymes and use this as a nitrogen source in fermentations. These fermentations are high volume or high value and should provide a significant new market for Manitoban peas and beans. The fermentation of glucose to ethanol by the yeast Saccharomyces cerevisiae was chosen as a model fermentation due to the size of the beverage and the fuel alcohol industries. Ethanolic fermentations are performed on the scale of 100,000 to 1,000,000 litres at a time. Last year 19 billion litres of (100%) ethanol were produced by yeast fermentations as transportation fuel alone. With slim margins the cost of raw material including nitrogen is paramount. Lactic acid fermentations were chosen due to the demanding nutritional requirements of lactic acid bacteria which usually require 6 to 8 essential amino acids, vitamins, and 3 or 4 different sources of these compounds in their fermentation medium. We are also investigating the use of pea and bean meal, pea protein and hydrolyzed pea protein in fermentations of Chinese Hamster Ovary (CHO) cells and with the yeast Pichia pastorius. These organisms represent producer organisms for the fermentation of recombinant proteins and monoclonal antibodies, representative of modern biotechnology processes. With success in substituting pulses, pulse meal, or pulse protein extract for current nitrogen sources in these fermentations Manitoba pulses will be positioned for their use in both current and new biotechnology fermentations.

PART 3: PROJECT ACTIVITIES AND PRELIMINARY RESULTS

Fermentation Microorganisms

We have obtained Lactococcus lactis, Lactobacillus plantarum, and Leuconostoc mesenteroides from the Biology Department of the University of Winnipeg. Only Lactococcus lactis is currently being used in lactic acid fermentations. We have obtained the yeast Saccharomyces cerevisiae from Coopers Brewery as a commercial beer yeast sold to home brewers. We also have access to a Pichia pastoris strain which we will use to determine the effect of pea protein on its growth.

Analytical

A BioRad Aminex HPX-87H column for HPLC analysis of substrates and products in the fermentations was purchased. This column is excellent analytical tool for this project since all alcohols, organic acids and sugars that are produced or consumed by microorganisms in virtually all important industrial fermentations can be identified and measured. We have also obtained the use of an HPLC system, an Agilent 1100, from the Chemistry Department. This machine is essentially ours, is already equipped with a refractive index detector so we do not have to constantly switch over detectors as would have been the case with the original HPLC system we planned to use and most importantly this system is equipped with an automatic sampling system. An autosampler means we can load up 20 or 30 samples at 5 o'clock and the sampler and HPLC will work unattended through the night and give us the results in the morning. This frees up manpower which would otherwise be needed just to sit by the HPLC and manually inject samples.

To use this HPLC system and column for the analysis of fermentations we need to calibrate with pure compounds and pure compounds in fermentation broth. We have completed standard curves for ethanol, lactic acid and glucose in the range of 0 to 5% Higher concentrations, which we will certainly see for glucose and ethanol, must be diluted into these ranges. A refractomenter is being used as a quick and approximate determination of the 3 compounds before dilution and analysis by HPLC. Ethanol, lactic acid and glucose are the 3 compounds we need to measure to determine whether the fermentations (ethanol and lactic acid) are proceeding and how quickly and what the effect of pulse proteins are.

Quantification of Nitrogen

The objective of this project is to determine whether pulse proteins can replace expensive nitrogen sources such as yeast extract and meat peptones. We have developed the Free Amino Nitrogen (FAN) assay using ninhydrin to determine the amount of amino acids present in any given fermentation broth. The FAN assay is widely used in the brewing and food processing industry to determine the levels of amino acids present. Microorganisms normally take up only amino acids and very short peptides from the medium as their nitrogen source. The assay also detects short peptides but that is not a problem for our work since yeast can metabolize short peptides and lactic acid bacteria, which are (weakly) proteolytic, can break down peptides into amino acids or take up short peptides. The FAN assay kit from Sigma Aldrich has been utilized in our work. A standard curve using glycine has been constructed.

Quantification of Nitrogen in Base Fermentation Media

It is difficult to obtain the details of fermentation medium composition from commercial sources (we have contacted Lallemand and Husky with no success). To begin this research we have used standard laboratory media for yeast and lactic acid bacteria fermentations. These media are Yeast Extract-Peptone Dextrose (YEPD) for yeast and de Man, Rogosa and Sharpe (MRS) for lactic acid fermentation. We have determined the amount of free amino acids in YEPD and MRS base media, both with and without proteolytic enzyme treatment to use as a comparator.

Proteolytic Digestion of Pulse Proteins

Early experiments have shown that commercially obtained pea flour and garbonza bean flour do not provide a great deal of useable nitrogen for either yeast or lactic acid bacteria when used directly. Neither yeast nor lactic acid bacteria are

very strongly proteolytic. To increase the amount of available nitrogen from these pulse flours we have attempted to digest them with proteolytic enzymes. The enzymes, Alcanase and Flavoraze, were obtained from Novozyme and are commercially used in the beverage, brewing, and food processing industry. When used at levels published in other research these enzymes increased the FAN levels of the flours. An increase of FAN was observed in the first 6 hours of proteolytic digestion at 50oC but little additional increase was seen upon longer digestion. Combining digestion with both enzymes simultaneously increased the release of FAN. We are currently awaiting information from the Canadian representative of Novozyme to determine the optimal conditions for this process, possible a different combination of proteases, whether we should include amylases and other fibre degrading enzymes to expose the protein to the proteases more effectively and, hopefully, to obtain some free enzymes.

In this regard we have obtained pure pea protein (Propulse) from Nutri-Pea Limited in Portage la Prairie. This protein is 82% pure in contrast to peas where protein is approximately 25%. Pure protein should be more easily digestible by proteolytic enzymes thus allowing us to determine more clearly the usefulness of pulses as a nitrogen source in commercial fermentations. We have contacted Burcon in Winnipeg for a sample of their Peazazz pea protein isolate and Clarisoy soy protein isolate.

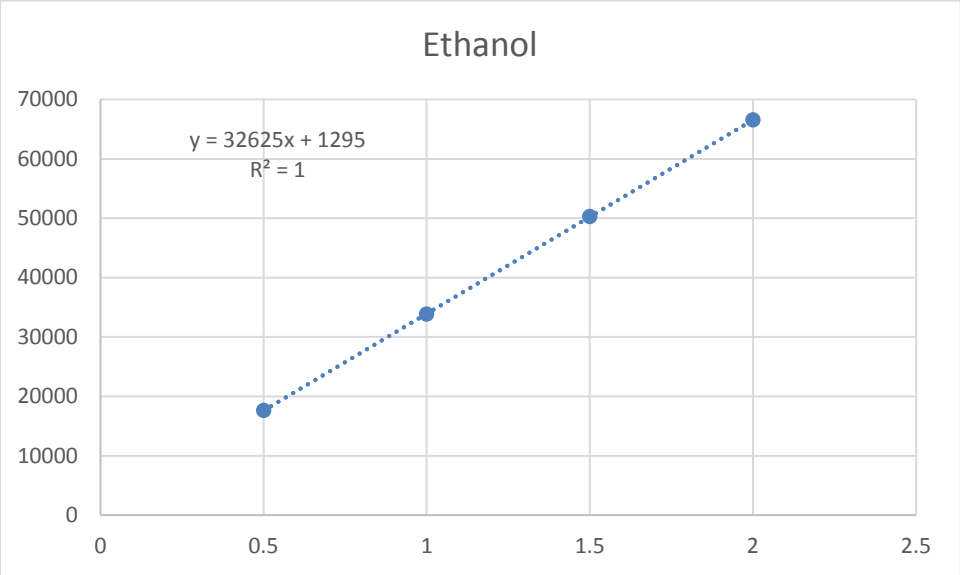
Nitrogen-Free Fermentation Media

We have manufactured nitrogen free versions of the base fermentation media YEPD and MRS. For yeast this is simply buffered glucose solutions but for lactic acid bacteria we have to make a buffered salt solution containing polysorbate 80, and citric and acetic acids but without the peptone, yeast extract and beef extract. These conventional nitrogen sources are provided at 25 g/L which gives an idea of the amount of relatively expensive nitrogenous material needed for a lactic acid bacteria fermentation (ie 250 kilograms for a 100,000 L fermentation, not an exceptionally large commercial fermentation). Both yeast and lactic acid bacteria grow extremely poorly in conventional media without nitrogen, not exactly rocket science but it gives us the baseline to determine the magnitude of growth and production of fermentation products with pulse proteins as the sole nitrogen source. We have conducted fermentations with several levels of glucose ranging from 50 to 200 g/L with similar results, without nitrogen being provided the microorganisms do not grow and ferment very slowly. Now we can add in pulse protein or proteolytically digested protein to determine how effective the pulse protein is at replacing conventional nitrogen sources. We can also determine how high a glucose concentration can be fermented to either ethanol or lactic acid with pulse protein as the nitrogen source. The higher the initial concentration of glucose, the higher the yield of the final product and the lower the cost of running the fermentation. There is also evidence plant proteins can protect microorganisms from the toxic effect of their own fermentation products. We can now see if this is true for pulse proteins.

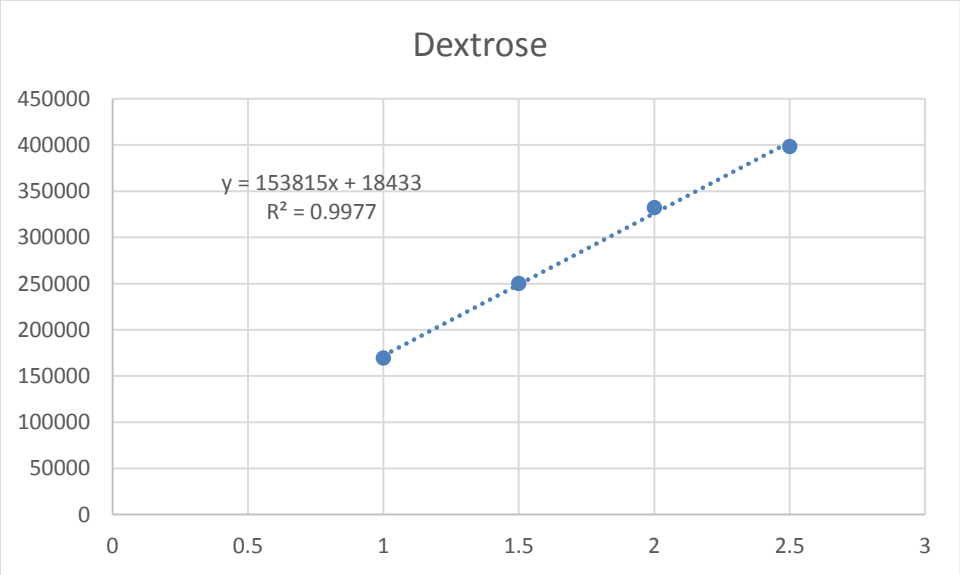
Conclusions

We have completed all the preliminary steps in the project except for using the newly obtained pure pea protein. All analytical and operational techniques have been worked out. We have baseline fermentations without nitrogen source and in conventional media for comparison. We have fermented both the lactic acid bacteria and the yeast at high glucose concentrations to more closely resemble commercial fermentations. The next steps are to determine how to most effectively hydrolyse pure pea protein, to determine how well it performs at industrial concentrations of substrates or even in real industrial substrates (Husky Energy, Minnedosa and Lallemand, Montreal) and to repeat these experiments with ground peas to determine whether with the right enzymatic treatment raw pea protein can also substitute for more expensive conventional nitrogen sources in commercial fermentations. We will also do these experiments for a wider variety of Manitoban pulse crops.

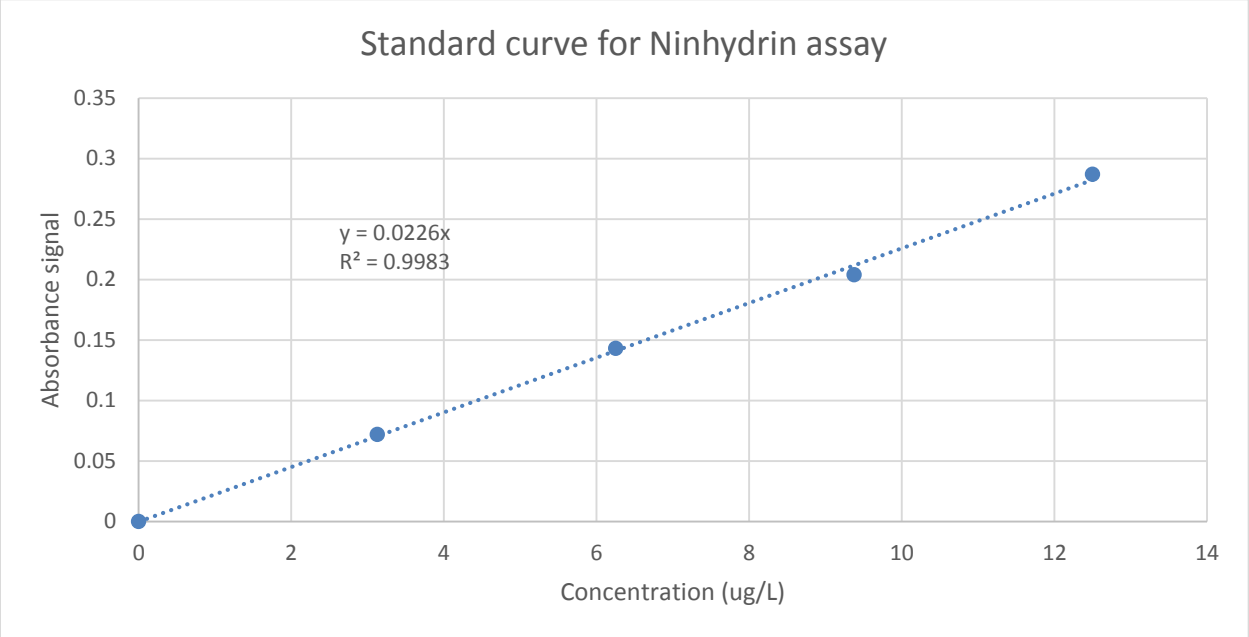
APPENDIX



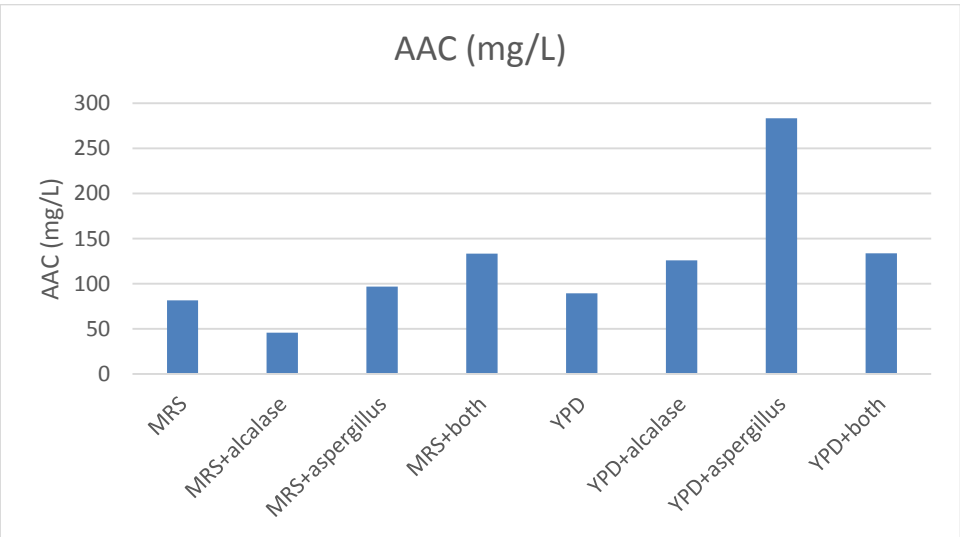
Calibration of the refractive index detector of the Agilent 1100 HPLC system using the Aminex HPX 87H column with 100% ethanol. Conditions were 0.5 mL/minute flow rate in 8 mM H_2SO_4 at room temperature.



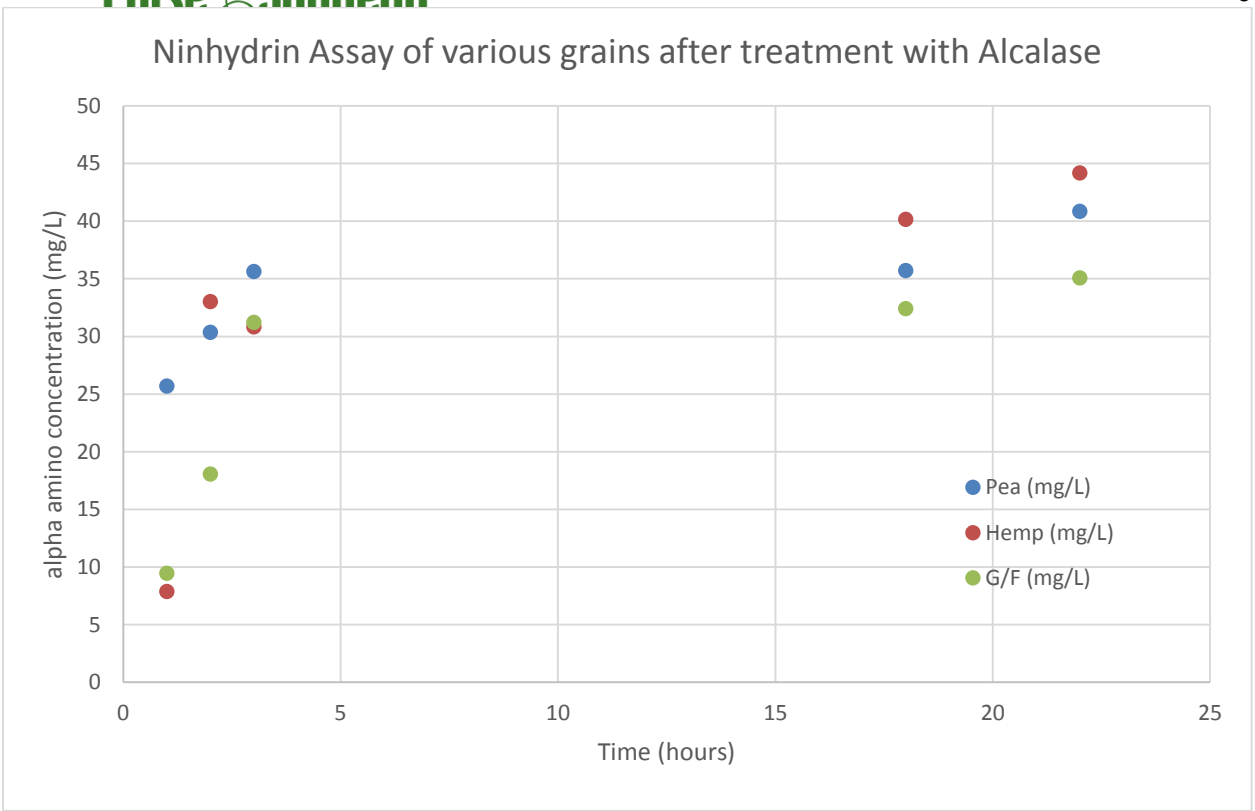
Calibration of the refractive index detector of the Agilent 1100 HPLC system using the Aminex HPX 87H column with dextrose (glucose). Conditions were 0.5 mL/minute flow rate in 8 mM H_2SO_4 at room temperature.



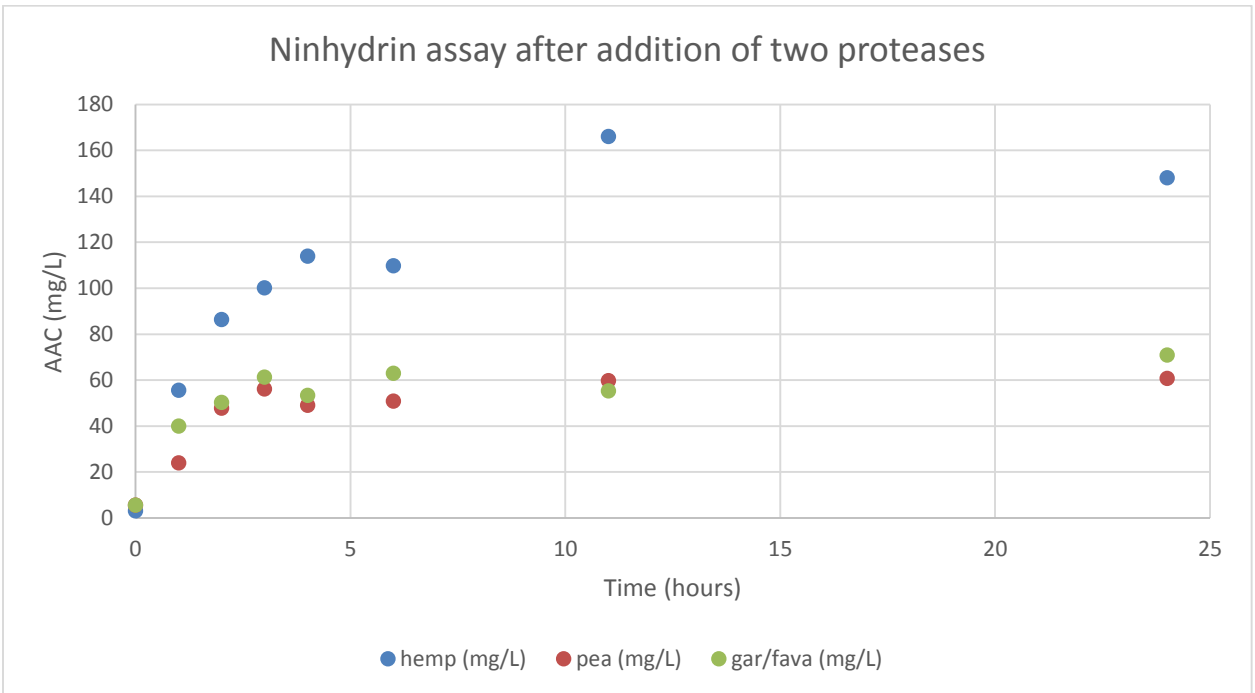
Quantification of free amino nitrogen (FAN) using the modified ninhydrin reagent. Glycine (50mg/mL) was used as the standard. Assays were conducted with the SigmaAldrich ninhydrin reagent kit N7295 using 1 mL of reaent and 2 mL of sample, diluted if necessary, boiled for 10 minutes, mixed with 5 mL of 95% ethanol and the absorbance was measured at 570 nm.



Free amino nitrogen (mg/L) as determined by the ninhydrin assay. MRS = de Man, Rogosa and Sharpe medium, YPD = Yeast Extract-Peptone Dextrose medium, alcalase = Alcalase protease from Novozymes (1 mL/100 mL, 24 hours at 50°C), aspergillus = Flavorase protease from Novozymes (1 mL/100 mL, 24 hours at 50°C), both = Alcalase plus Flavorase proteases (1 mL of each/100 mL, 24 hours at 50°C). Media prepared according to manufacturers instructions.



Proteolytic digest of pulse proteins with Alcalase (Novozymes) using1 mL enzyme/100 mL protein solution, 24 hours at 50°C. Increase in the free amino nitrogen content (mg/L).



Proteolytic digest of pulse proteins with combined Alcalase and Flavorase (Novozymes) using1 mL of each enzyme/100 mL protein solution, 24 hours at 50°C. Increase in the free amino nitrogen content (mg/L).