Enzyme-Mediated Conversion of Glucolimnanthin to Bioherbicidal Breakdown Products

By

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An Undergraduate Thesis Submitted to
Oregon State University

In partial fulfillment of
the requirements for the
degree of

Baccalaureate of Science in Bioresource Research,
Sustainable Ecosystems

May 16, 2012

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Abstract

The oilseed crop *Limnanthes alba* (white meadowfoam) is grown in Oregon. Methods were developed for the production of a liquid-based bioherbicide from meadowfoam seed meal (MSM). It was determined that glucosinolate-containing extracts of MSM can be combined with active-enzyme-containing liquid extracts of meadowfoam seed to form a fermented product rich in bio-active glucosinolate breakdown products (GBPs). It was discovered that the relative amounts of glucosinolate breakdown products formed were dependent upon the extraction solvent for MSM, the extraction solvent for seed, and the fermentation matrix. Aqueous extraction and fermentation led to formation of products rich in the isothiocyanate (ITC) product, while addition of the organic solvent ethanol to these matrices caused a relative increase in the breakdown of glucosinolate to the nitrile product.

List of Abbreviations

ACN: acetonitrile

EtOH: ethanol

GBPs: glucosinolate breakdown products

GLN: glucolimnanthin

ITC: 3-methoxybenzyl isothiocyanate

MeOH: methanol

MSM: meadowfoam seed meal

Nitrile: 3-methoxyphenylacetonitrile

TFA: trifluoroacetic acid

Introduction

Organic farming is a rapidly growing industry. Between 2001 and 2011 the global number of hectares devoted to organic agriculture multiplied by 2.3 (1). An effective practice in organic farming is to utilize allelopathic compounds, the naturally occurring secondary metabolites of some organisms that negatively or positively affect other organisms (2). Bioherbicides, agricultural phytotoxins not comprised of synthetic chemicals, are a commonly employed allelopathic tool. The demand for environmentally friendly bioherbicides is increasing to match these agricultural interests. Various plants of the order Brassicales are known to have bioherbicidal activity (3-6). A source of bioherbicidal compounds in Oregon is *Limnanthes alba* (white meadowfoam), an oilseed crop of the family Limnanthaceae within the order Brassicales (7, 8). Meadowfoam seed is composed of approximately 24-28% oil and 21-25% protein by weight (9). The oil is extracted from seed in a large-scale industrial process. A by-product of this extraction is the ground extracted seed known as meadowfoam seed meal (MSM), which currently has limited commercial value.

The glucosinolate, glucolimnanthin (GLN), is found in white meadowfoam seed and MSM. Glucosinolates are also found in commonly cultivated vegetables of the order Brassicales such as cabbage, Brussels sprouts, broccoli, and cauliflower (10). Glucosinolate breakdown products (GBPs) have been shown to have biological activity (11). For example, the isothiocyanate, sulforaphane, originating from the glucosinolate, glucoraphanin, in broccoli and cauliflower has been studied as a cancer chemopreventive (12, 13). The enzyme myrosinase is also present in white

meadowfoam, and is capable of catalyzing the degradation of GLN to form two bioherbicidal metabolites, namely 3-methoxyphenylacetonitrile (nitrile) and 3-methoxybenzyl isothiocyanate (ITC). However, active myrosinase is not present in MSM, since the heat-labile myrosinase loses enzymatic activity during the heated industrial extraction process that yields oil from seed.

A method has been developed to metabolize GLN to nitrile and ITC using the myrosinase enzyme. A very small amount of seed (1% w/w) as a source of active myrosinase is sufficient for complete conversion of GLN in MSM to the GBPs nitrile and ITC (14). The resulting GBP-rich MSM (augmented MSM) is a more potent herbicide than MSM, as demonstrated by its effect on coleoptile emergence of *Bromus tectorum* (downy brome) (14). Previous studies pointed to the nitrile metabolite as the active herbicidal ingredient in meadowfoam (15). This was later confirmed when GLN, nitrile, and ITC were individually assayed for herbicidal activity (14).

Although augmented MSM has improved herbicidal activity as compared to MSM, there are limitations. A solid augmented MSM product may be somewhat cumbersome to store, ship, and apply to agricultural fields. The shelf-life of augmented MSM is limited, likely due to the reactivity of GBPs, especially ITC, which presumably could react with protein present in MSM. One way to potentially avoid some of these limitations would be to develop a spray-able liquid product. A GLN-containing extract of MSM could be combined with a myrosinase-containing extract of meadowfoam seed as needed prior to herbicide application. Keeping these extracts segregated until use could potentially prevent the GBP loss associated with storage.

The present research focused on methods for the production of such a liquid formulation. Project goals included the development of methods to extract GLN from MSM, to extract myrosinase from seed, and to combine and ferment these two extracts together to efficiently produce GBPs (Fig.1). These experiments were conducted in Dr. Stevens' laboratory in Weniger Hall of Oregon State University.

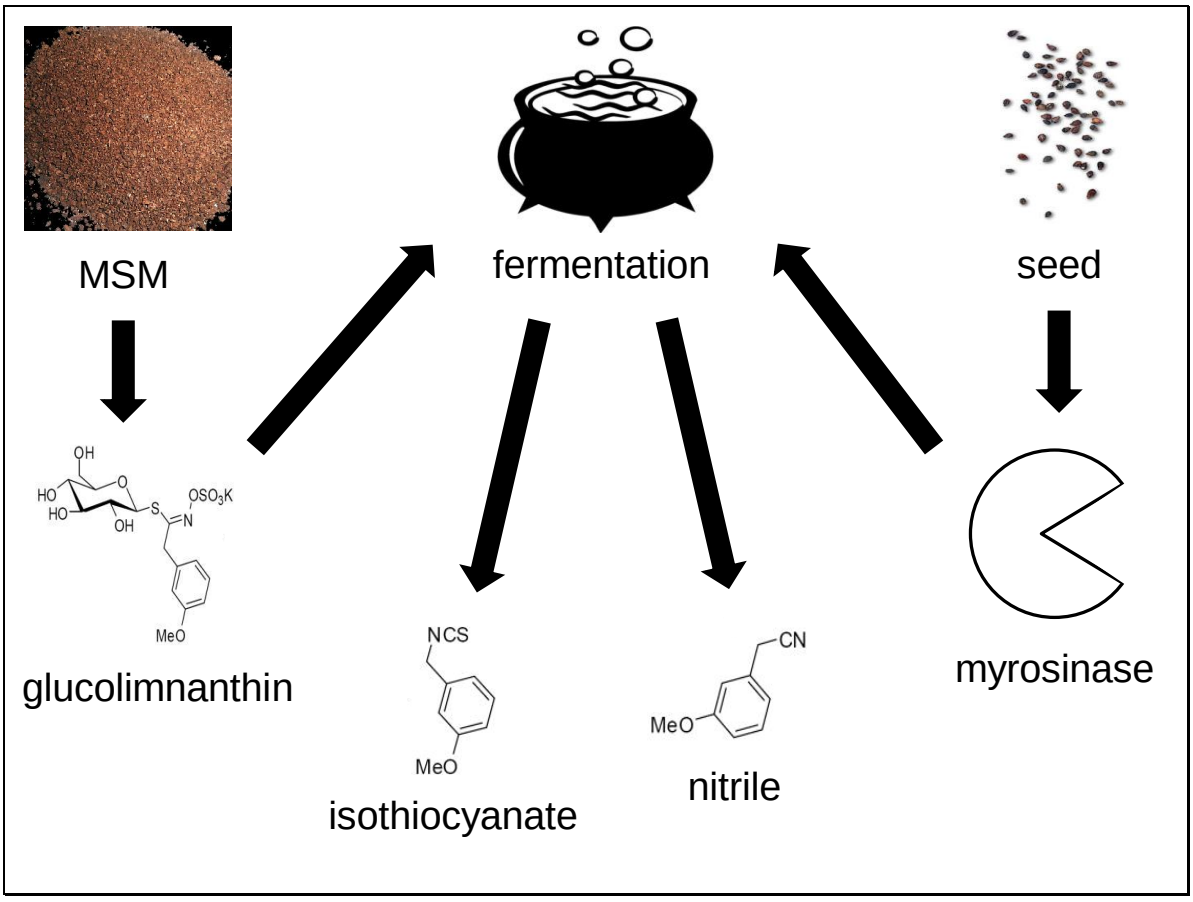

Fig 1. Visual representation of proposed method for production of GBPs.

Materials & Methods

Extraction of GLN from MSM

Various solutions of water and ethanol (EtOH) were used in the extraction of glucolimnanthin (GLN) from meadowfoam seed meal (MSM) in most experiments. This choice was made early in the project due to preliminary findings that EtOH solutions were superior to methanol (MeOH) solutions for extracting GLN from MSM while avoiding extraction of other unwanted components, such as proteins. Successful GLN extraction procedures included “batch” extractions (adding MSM to stationary extraction solvent and allowing extraction to take place for a specified amount of time) and “serial” extractions (adding MSM to stationary extraction solvent as before, then carefully replacing the solvent with fresh solvent at specified intervals using a Pasteur pipette). Batch MSM extractions were usually terminated after two hours since batch extraction in excess of two hours did not significantly increase yields.

For batch extractions, 30 mL solvent was mixed with 6 g MSM in 50 mL plastic screw-capped tubes. Each tube was vortexed for 10 seconds and allowed to stand for two hours with an additional 10-second vortex every 30 minutes. The tubes were then centrifuged for ten minutes at 4000 rpm and supernatants were carefully removed using a Pasteur pipette. Serial extractions were performed with 0.5 g MSM mixed with 10 mL solvent in 15 mL glass tubes. Tubes were vortexed for 10 seconds, and then extractions proceeded for one hour with an additional 10-second vortexing every 30 minutes. The tubes were centrifuged for ten minutes at 4000 rpm. As much supernatant as possible was removed with a Pasteur pipette and the volume removed was recorded. The

solvent was replaced and the extraction procedure was repeated for each additional serial extraction. All MSM extractions took place at room temperature.

Extraction of Myrosinase from Seed

Solutions of water and EtOH and solutions of water and sodium chloride (NaCl) were used to extract enzyme from seed. Concentrations are abbreviated as “% EtOH” rather than “% v/v EtOH/water” and "% NaCl" instead of "% w/v NaCl/water". The “batch” method was chosen for the extraction of enzyme from seed and was used in all myrosinase extractions. Seed extraction took place by grinding 15 g meadowfoam seed for two minutes in a single-setting Black & Decker coffee bean grinder. One gram ground seed and five mL solvent were mixed in a 15 mL glass tube. The tubes were vortexed for ten seconds, and then extractions proceeded for two hours with an additional 10-second vortex every 30 minutes. Tubes were centrifuged for 10 minutes in a clinical centrifuge on setting six. The myrosinase-containing supernatants were removed using a Pasteur pipette. Seed extraction was performed at room temperature.

Fermentation of the GLN-containing MSM extract

Fermentation of the precursor compound GLN with myrosinase produces the nitrile and ITC breakdown products. The sources of GLN and myrosinase in this combination are the liquid MSM extract and liquid seed extract, respectively. A ratio of 20 µL seed extract per mL MSM extract was used for conversion of GLN present in MSM extract to nitrile and ITC breakdown products over a 23-hour period in various fermentation matrices.

Since both the MSM and seed extracts were always prepared using a ratio of 5 mL solvent per gram solid (except in the cases of serial MSM extraction and enzyme stability experiments), 20 µL seed extract per 1mL MSM extract generally corresponded to a 2% weight basis addition of seed to MSM. An equal volume of either water or EtOH/water solution was added to the MSM extract prior to the addition of seed extract. The purpose of this solvent addition was to maintain the total percentage EtOH in the fermentation matrix constant, while observing the effect of differing MSM extraction solvents, or vice versa. As a positive control, ground seed (no extraction involved) was used as a myrosinase source in some experiments. Fermentations always occurred at room temperature.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is the method of sample analysis that was chosen to determine the type and amount of products that were extracted and/or formed as a result of any given extraction and/or fermentation. HPLC instrumentation first separates the analytes within a sample on the basis of polarity by pumping them through a chromatographic column. As the separated sample elutes (exits the column), analyte identity is determined by a photodiode-array detector, a device that emits a specified spectrum of light and detects which wavelengths of light are absorbed. Analyte concentration is calculated based on how much light is absorbed at those wavelengths.

The term “stationary phase” is used in conjunction with many quantitative analytical techniques and instruments. As it applies to HPLC, the stationary phase

refers to the chromatographic column and the type of beads packed within. Bead size, bead coating, density of bead packing, column length, and column diameter are important factors relevant to the purpose and effectiveness of the stationary phase. The reversed-phase chromatography method was used for sample analysis, meaning that the stationary phase was relatively nonpolar and the mobile phase was relatively polar. These nonpolar hydrocarbon-coated bead assemblies attract nonpolar substances more strongly than polar substances. Therefore, when a liquid sample consisting of analytes of varying polarities is injected into the reversed-phase chromatography column, the nonpolar analytes are retained longer in the bead-packed column whereas the more polar analytes elute first. This common analytical method allows for clean separation of the GLN, nitrile, and ITC peaks due to differences in analyte polarities. The most polar GLN elutes first, followed sequentially by nitrile and ITC.

The term “mobile phase” as it applies to HPLC refers to the liquid (eluent) that passes through the bead-packed column. This mobile phase generally consists of water (in this case 0.1% trifluoroacetic acid (TFA) in water), one of many water-miscible organic solvents (in this case acetonitrile (ACN)), and the analyte-containing sample injection. With little exception, all experimental samples were prepared and analyzed in duplicate. This was to account for the inevitable experimental variation in observed peak areas and to estimate the experimental error.

The eluent is introduced once the computer queue initiates an injection. Isocratic elution is often used, meaning that there is a constant ratio of water to the less polar organic solvent. However, for purposes of obtaining better resolution of polar and apolar analytes gradient elution was chosen for the detection and quantitation of compounds in

this investigation. Gradient elution means that an increasing percentage of ACN is added to the ACN/water mixture as the injected sample progresses through the column. This method produces sharper peaks because the eluent mixture becomes less polar as more ACN is introduced. As the eluent polarity decreases, the relative affinity of analyte for the mobile phase is increased. This prevents “tailing”, the elution and detection of diminishing amounts of analyte after the majority of the analyte has eluted, allowing for taller and narrower peaks.

Sample Analysis

The instruments chosen for qualitative and quantitative analyte detection were a Waters 600 HPLC device coupled with a Waters 2996 photodiode-array detector monitoring at an absorption wavelength of 274 nm and a Waters 717 autosampler. The Phenomenex LiChrospher® 5 µm RP-18 100 Å 250 x 4 mm was the reversed-phase liquid chromatography column chosen for these analyses. The developed method used a 10 µL sample injection volume and a constant flow rate of 1 mL/minute. Gradient elution of the column was employed, using 0.1 % trifluoroacetic acid (TFA) as the polar solvent and acetonitrile (ACN) as the non-polar solvent. ACN was increased from 5% to 100% over the first 30 minutes, and then lowered from 100% back to 5% from 30 to 32 minutes. Before a new sample injection was initiated, a mobile phase consisting of the original ACN/water ratio (5% ACN) was pumped through the column for an appropriate equilibration period. The time chosen for re-equilibration with 5% ACN was 13 minutes, for a total injection method time of 45 minutes per sample. Samples were diluted to a

range of .01 to 1 mg analyte per mL sample, an effective quantitation range for the instrumentation.

The external standard method of calibration and linear regression of analyte peak areas were used to determine analyte concentration. The external standard calibration method specifies that a standard curve of known concentrations of GLN, nitrile, and ITC were analyzed before and after the actual samples from any given experiment. The standard curve included seven standard mixtures of increasing concentration, all of which were prepared from the same original standard mixture containing the three analytes. The purpose was to obtain absorbance values for concentrations of the analytes well below and above the values expected from experimental samples. This analytical technique provided great accuracy in determining the analyte concentrations within the extraction and fermentation samples through interpolation rather than extrapolation of the standard curve.

Variation in response during the time required to run the HPLC computer's entire queue of experimental samples was minimal. Over the course of a large sample set (about 60 injections including external standards) GLN and nitrile exhibited a variation of response up to 2%, whereas the relatively less stable ITC showed variation as high as 10%. A method utilized to verify accuracy of quantitation involved the placement of a standard curve at the beginning and end of an entire sample set.

As a verification of stability, repeat standards were often introduced at specified intervals, such as after every ten samples injected. Samples were often included in these repeat injection sets in addition to standards. This was done to observe and account for differences in analyte loss between samples and standards as the result of

any matrix component besides the analytes within experimental samples that may have affected analyte stability. Such differences were never observed to an extent that would mandate correction. The protein content of MSM was analyzed using the method of Lowry, et al. (16).

The spreadsheet program Microsoft Excel 2007 was utilized to reduce and interpret data. This effort yielded information such as moles GLN obtained per gram MSM extracted, percent conversion of extracted GLN to nitrile and ITC, and the effect of introduced experimental variables upon these values. Findings throughout the investigation were graphed using the programs Microsoft Excel 2007 and GraphPad Prism 5.

Results & Discussion

MSM Extraction

One of the first attempts at optimizing bioherbicidal compound yield was determination of the most thorough and efficient method for obtaining the precursor compound GLN from solid MSM. A logical initial consideration was maximization of MSM surface area during extraction. Although the solid MSM provided by agricultural industry for research purposes was already quite fine as a result of oil extraction, an early experiment involved an attempt to reduce particle size even further to obtain additional GLN. Utilizing a single-setting coffee grinder, the MSM was ground for 2 minutes. An experiment was conducted comparing GLN yield from ground vs. unground MSM.

Increasing surface area prior to extraction in pursuit of increased GLN yield suggested there was no notable difference in GLN yields between ground vs. unground MSM (Fig. 2), at least not when additional grinding is conducted for only 2 minutes in a small coffee bean grinder. It should be noted that there was a visible difference in the particle size of MSM before and after grinding, indicating that more surface area was becoming exposed. This suggests that even more grinding would produce similar results, having little to no effect on GLN yield.

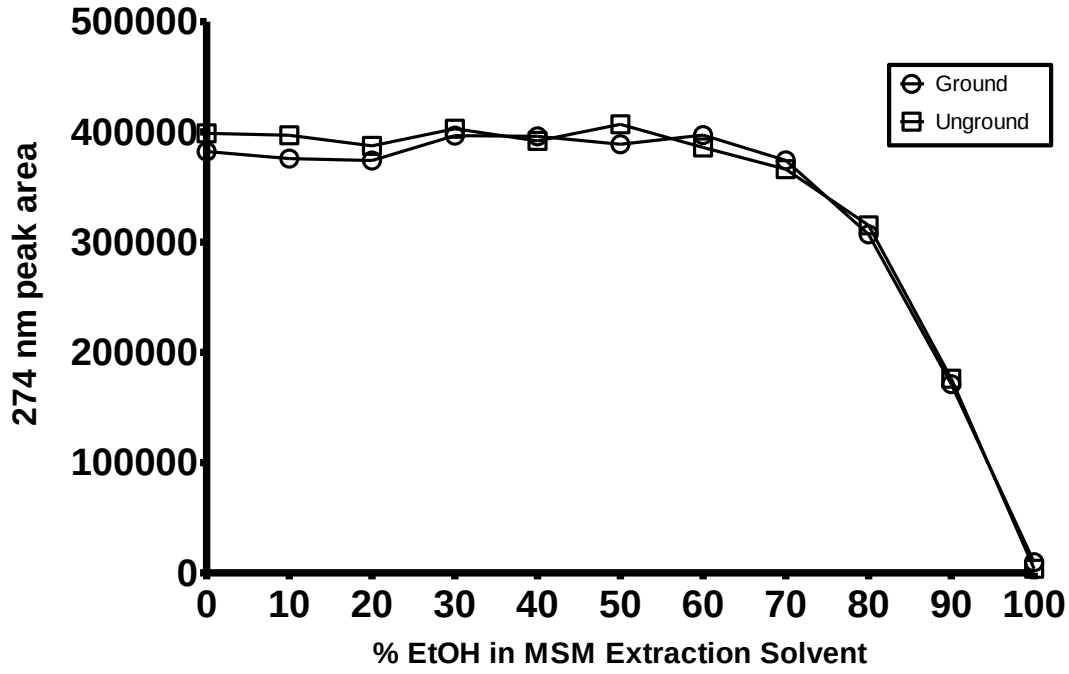

Fig 2. Extraction of GLN from ground vs. unground MSM. 0-100% EtOH was the solvent used in the 2-hour extraction.

The extraction of GLN from MSM occurs quite readily with various percentages of MeOH or EtOH in water, but an experiment comparing 0-100% of each alcohol as an extraction solvent revealed what were previously unapparent disparities. Findings showed EtOH and MeOH to be almost exactly as effective as one another at extracting GLN from MSM at most percentages of alcohol in water (Fig. 3). However, EtOH extracted much less protein than MeOH at every explored point between 0-100% alcohol (Fig. 4).

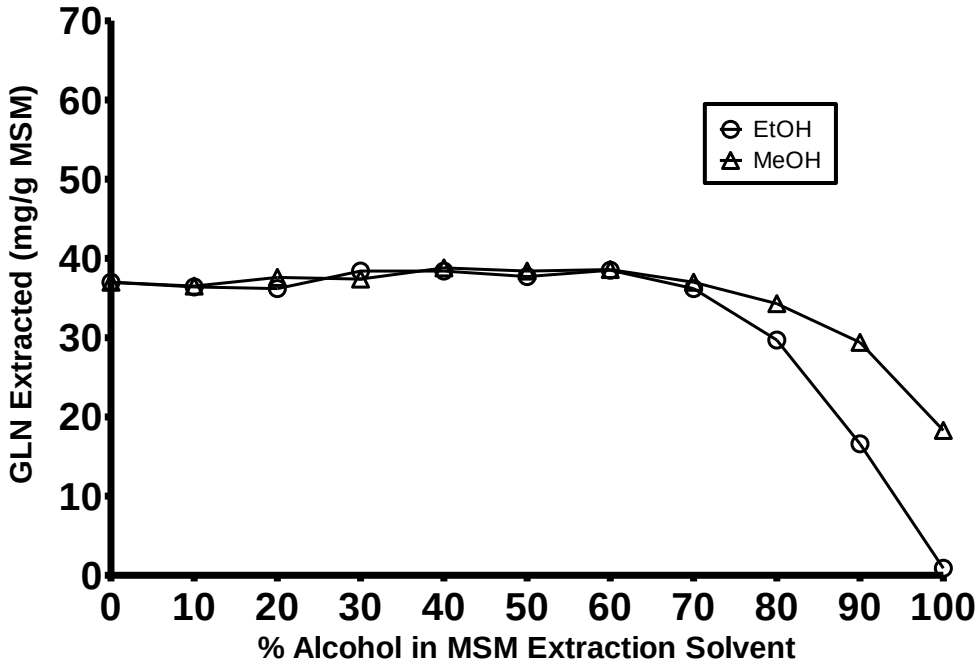

Fig 3. Extraction of GLN from MSM. 0-100% MeOH and EtOH were the solvents used in the 2-hour extraction.

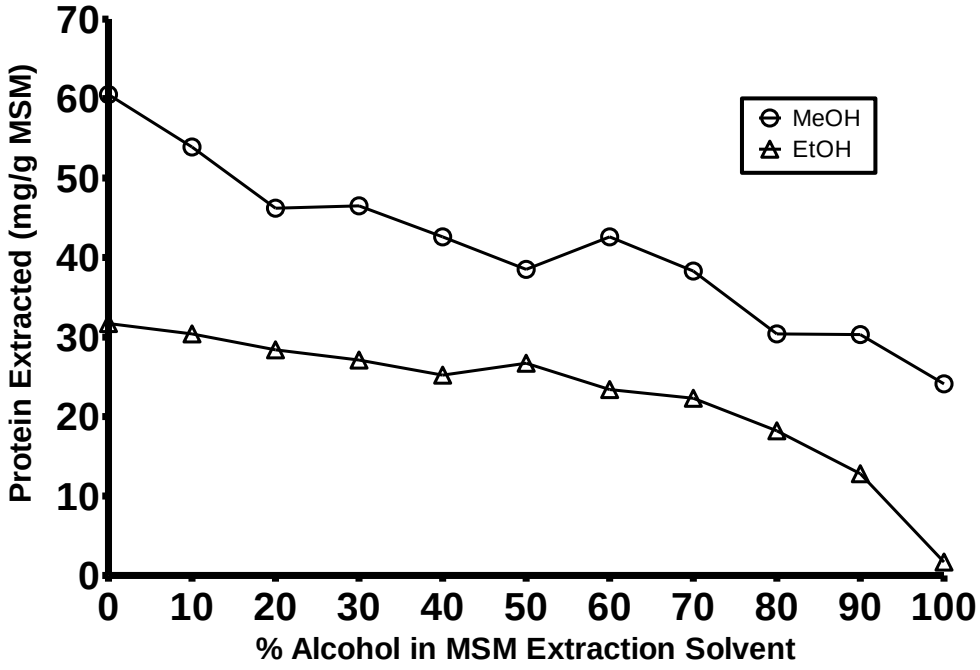

Fig 4. Extraction of protein from MSM. 0-100% MeOH and EtOH were the solvents used in the 2-hour extraction.

These findings reveal EtOH as the more desirable extraction solvent of the two alcohols for multiple reasons. When conducting an extraction, it is the aim to obtain an extract that is as homogenous as possible, while excluding unwanted substances. It is for this goal that extractions of various types were explored. The desire for extracting GLN exclusively, to the greatest reasonable degree, was a consideration in all MSM extractions, despite the fact that GLN was only one of multiple components being retained by solvent. This common goal of extract homogeneity alone justifies designating EtOH as the more efficient solvent than MeOH.

A second noteworthy incentive is to leave as much protein as possible behind during GLN extraction. MSM is feasibly suitable as livestock feed, but the presence of the relatively unhealthy GLN is a deterrent, as would be a deficiency of protein. MeOH would therefore prove less effective than EtOH once more, with the additional goal of eventually allowing for the production of viable livestock feed in mind. EtOH is also less toxic than MeOH. In any case, EtOH was the clear choice between the two cheap and easily obtainable solvents to utilize for investigation proceedings.

A logical curiosity was the determination of a timeline detailing the progression of GLN extraction using the choice solvent EtOH. Such a timeline served to detail the point at which it might be prudent to terminate a “batch” extraction. Also of interest was the extraction of one of the desired bioherbicidal breakdown products, nitrile, present in MSM. The timeline experiment suggests that a batch extraction of GLN and nitrile is complete after only 2 hours (Fig. 5, 6). Therefore, subsequent batch extractions lasted two hours.

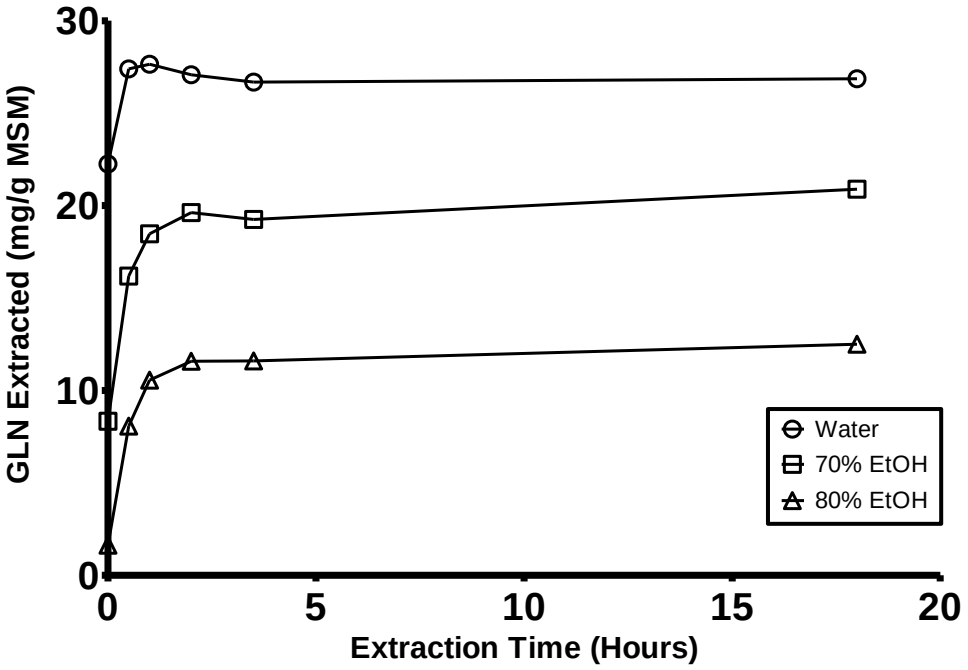

Fig 5. Time course of GLN extracted from MSM using 0, 70, and 80 % EtOH as solvents in the 18-hour extraction.

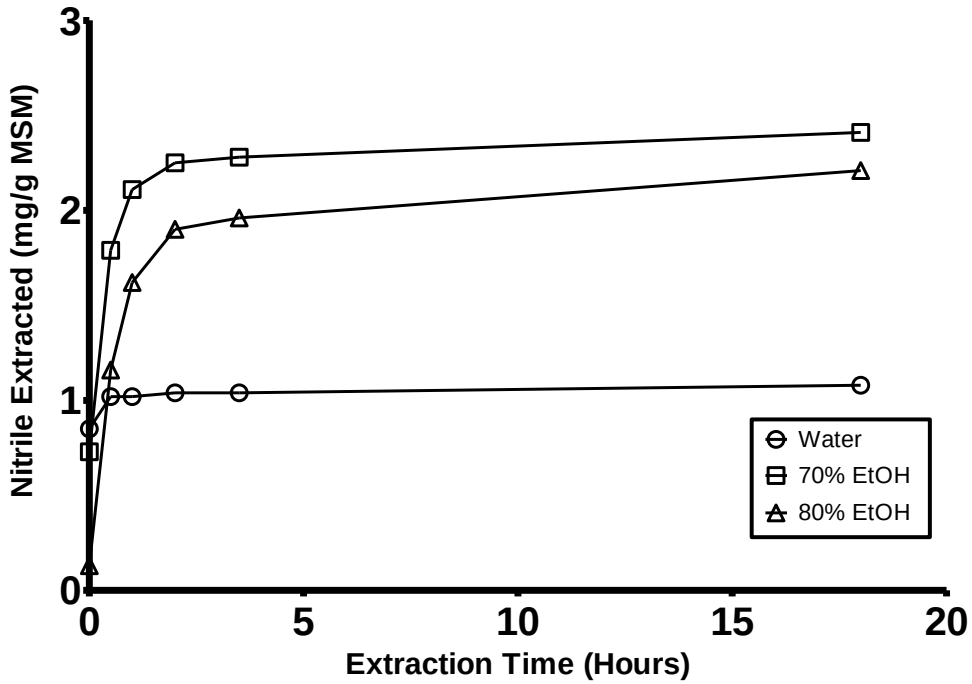

Fig 6. Time course of nitrile extracted from MSM using 0, 70, and 80 % EtOH as solvents in the 18-hour extraction.

The findings indicate differing ideal solvents for the extraction of these two compounds, likely due to the differences in polarity. Optimizing GLN extraction was the priority, as nitrile is present in much smaller quantities than GLN (roughly 10% relative) within MSM (Fig. 5, 6). While the greatest amount of total GLN recovered was observed using water (Fig. 5), the greatest ratio of GLN extracted to protein extracted was observed using 70% EtOH as solvent (Fig. 3, 4). This narrowed the focus of MSM extraction solvent to a range of 0-70% EtOH for the remainder of the investigation.

The effectiveness of multiple extractions with fresh solvent (serial extractions) was investigated using 30-70% EtOH as extraction solvent. Results regarding which %EtOH was the most efficient exhibited some experimental variation as evidenced by an unlikely nonlinear trend. This is of little consequence, as extraction trends were already established in prior experiments. The most novel observation of this experiment is not compromised by such variation. This observation is that each successive serial extraction, regardless of %EtOH in solvent, yields between a third and a fourth of the amount of GLN or nitrile that was obtained in the extraction prior (Fig. 7, 8). This type of finding is very important in an optimization investigation. In a large-scale extraction, the determination of a point at which additional time and solvent invested are no longer worth the GLN and nitrile yields is a fundamental consideration.

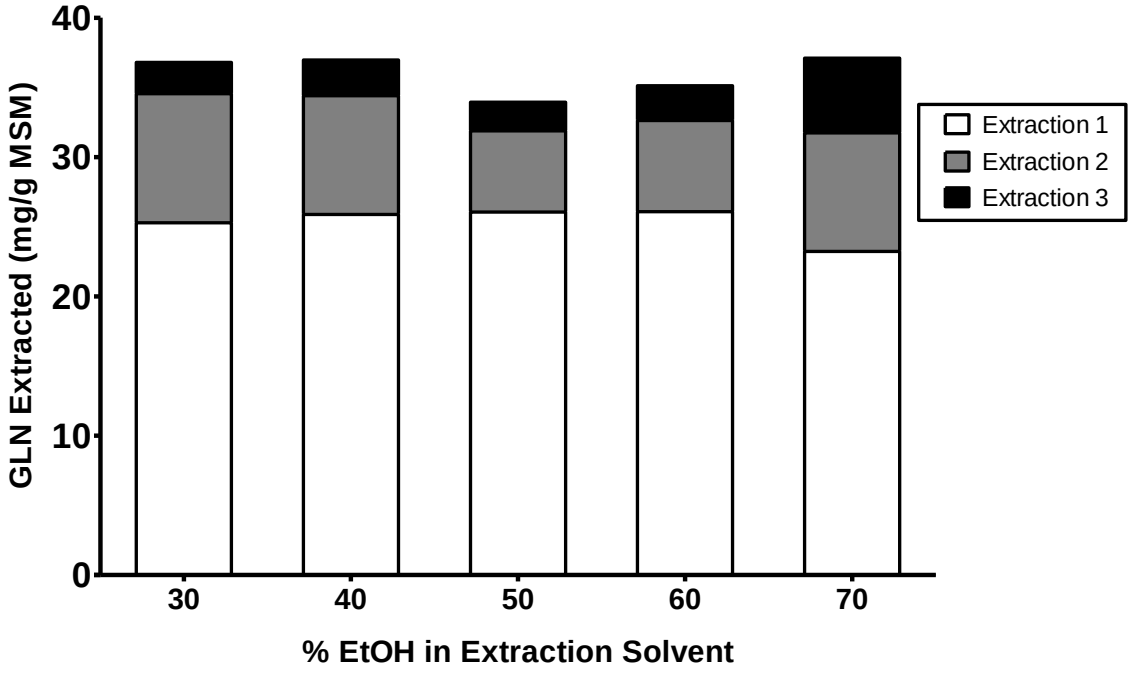

Fig 7. Serial extractions of GLN from MSM using 30-70% EtOH as solvents in three 1-hour extractions.

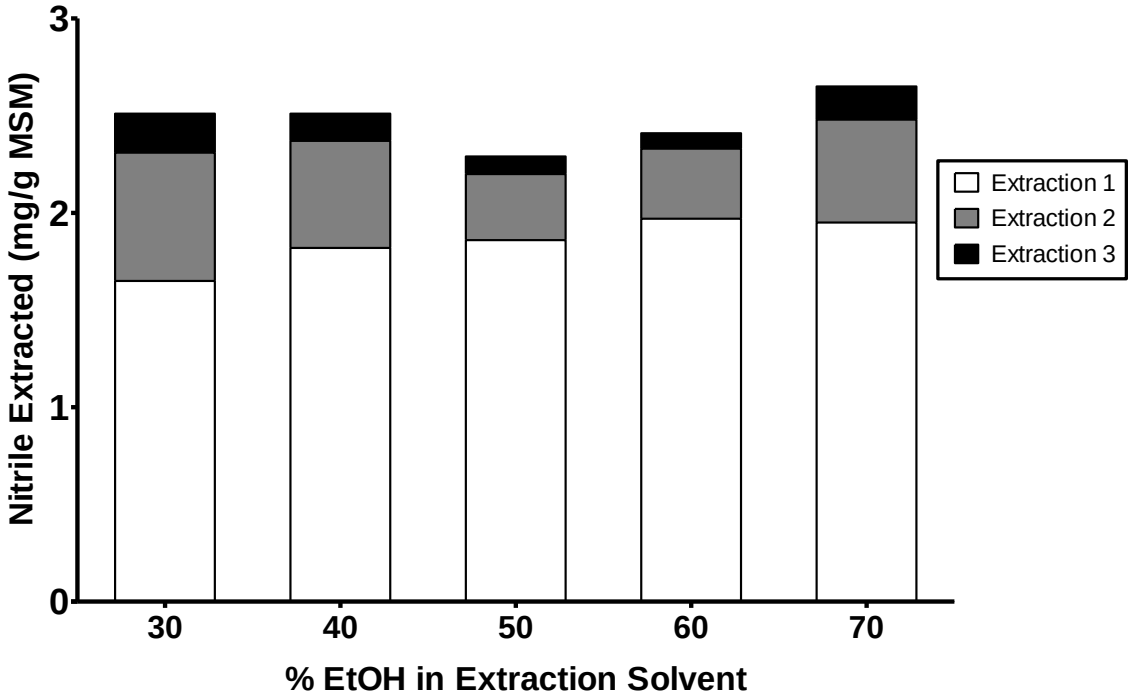

Fig 8. Serial extractions of nitrile from MSM using 30-70% EtOH as solvents in three 1-hour extractions.

Seed Extraction

EtOH and NaCl were explored as economical additives to the aqueous seed extraction solvent for the purpose of more effectively extracting active myrosinase. The optimum additions of EtOH and NaCl were found to be 20% (Fig. 9) and 2% (Fig. 10), respectively. Although the amount of myrosinase activity was similar between 2% and 4%, there was no logical need to add more NaCl than necessary to a product that may end up on an agricultural field. Myrosinase extracted with NaCl thereafter was prepared using 2% NaCl as extraction solvent. Seed extract prepared with EtOH used the optimized concentration of 20% EtOH in solvent for the remainder of the investigation.

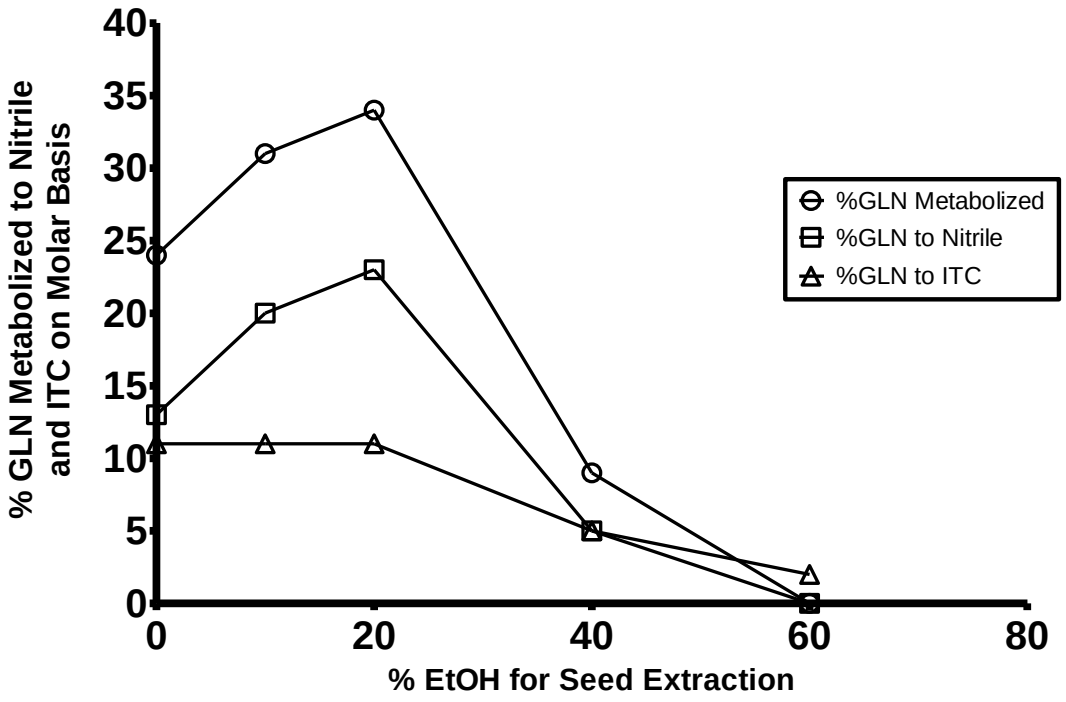

Fig 9. Effect of % EtOH in myrosinase extraction solvent on GLN conversion. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. The 23-hour fermentation was performed in 35% EtOH.

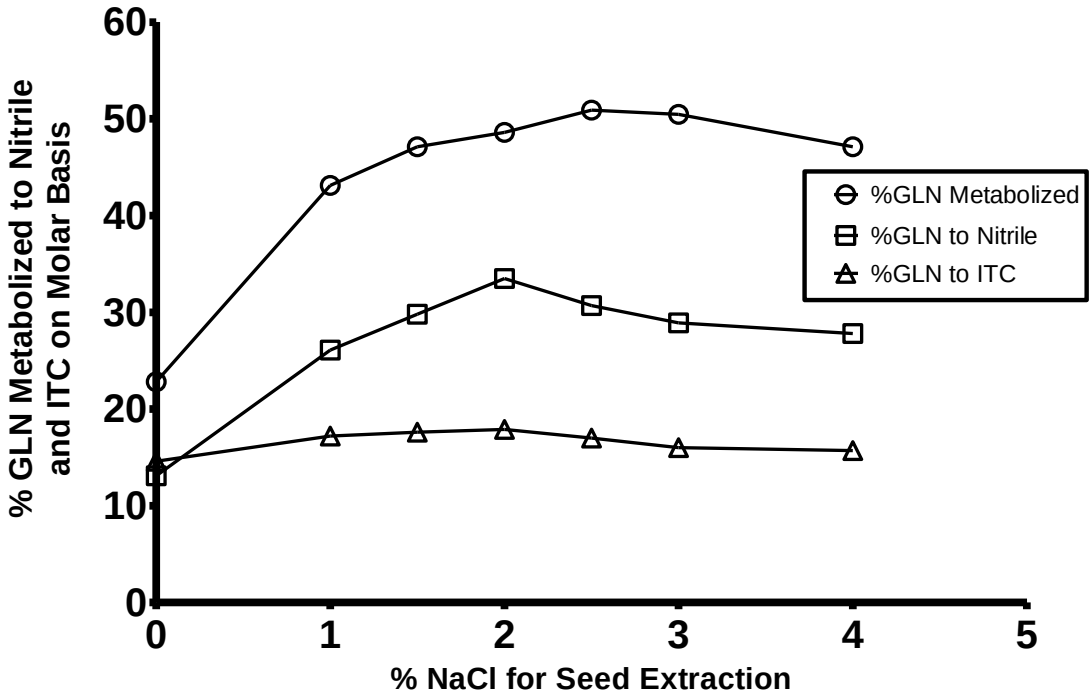

Fig 10. Effect of %NaCl in myrosinase extraction solvent on GLN conversion. 1-4% NaCl was used as solvent in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. The 23-hour fermentation was performed in 35% EtOH.

An experiment was performed to determine the minimum ratio of seed to MSM required for complete metabolism of GLN, so as to not use more than was absolutely necessary since oil-containing seed is sacrificed during extract creation. This was done by progressively diluting a 20% EtOH myrosinase extract (0.02 g seed per g MSM) and monitoring the effect on GLN metabolism. Ratios from 0.005 to 0.0017 were used based on the results of a previous range-finding experiment (data not shown). A ratio of 0.005 g seed per g MSM was effective, metabolizing 94% of the GLN (Fig. 11). Further dilution of the enzyme extract metabolized progressively less GLN.

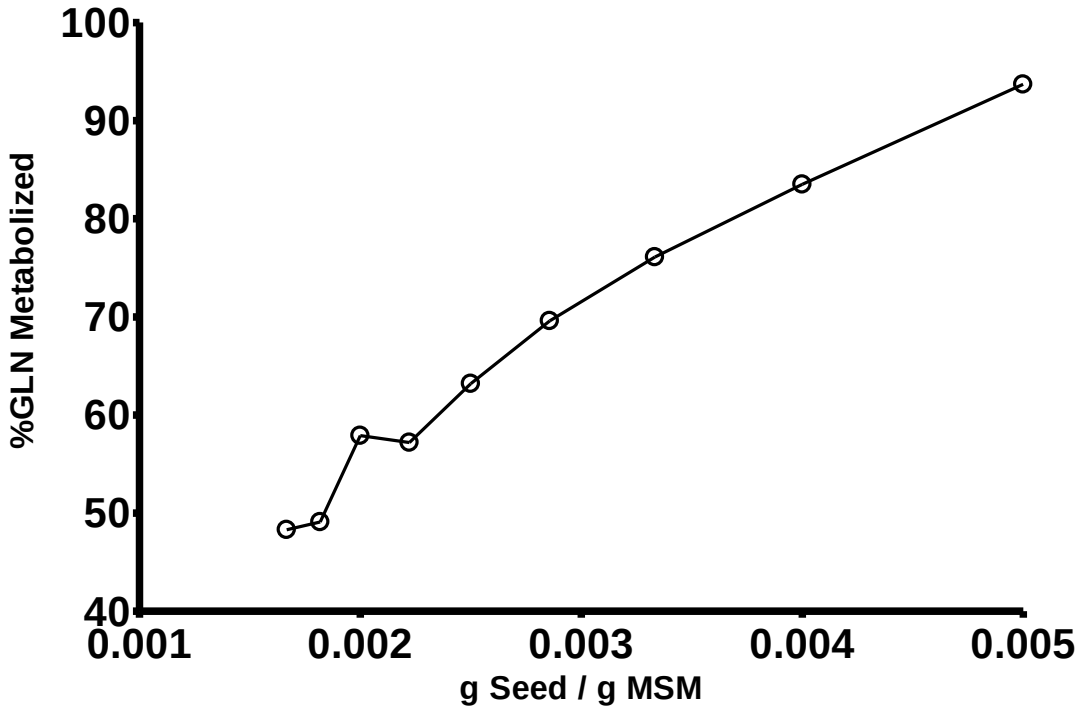

Fig 11. Effect of seed/MSM ratio on GLN metabolism. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with water. The 23-hour fermentation was performed in water.

An enzyme extract characteristic that is perhaps as critical as potency is stability. Investigation of the shelf-life of the 20% EtOH and 2% NaCl myrosinase extracts began with a dilution that converted less than 100% of GLN under investigated fermentation conditions on the day of extract creation. If storage began with an enzyme concentration that converted 100% of GLN during the initial fermentation, and then converted 75% after 122 days, it would not have been clear at what point in time the enzyme lost enough activity to convert less than 100% of GLN present during fermentation. For this purpose we chose to start off with a myrosinase extract dilution that would convert roughly 60% of GLN before storage began (Fig. 11), created using a ratio of 0.0025 g seed per g MSM.

Stability of the 20% EtOH extract at both 25°C (room temperature) and 4°C (refrigerator temperature) in the dark was explored. After 122 days of storage in 25°C, 35% of the original enzymatic activity remained, and after 122 days of storage in 4°C, 73% of the original enzymatic activity remained (Fig. 12). Storage of the 2% NaCl extract at both temperatures in the dark was also investigated. After 122 days of storage in 25°C, 72% of the original enzymatic activity remained, and after 122 days of storage in 4°C, 84% of the original enzymatic activity remained (Fig. 13). It is unknown if it would be more effective to store the extracts at an even lower temperature, as freezing may compromise the myrosinase activity within liquid extracts.

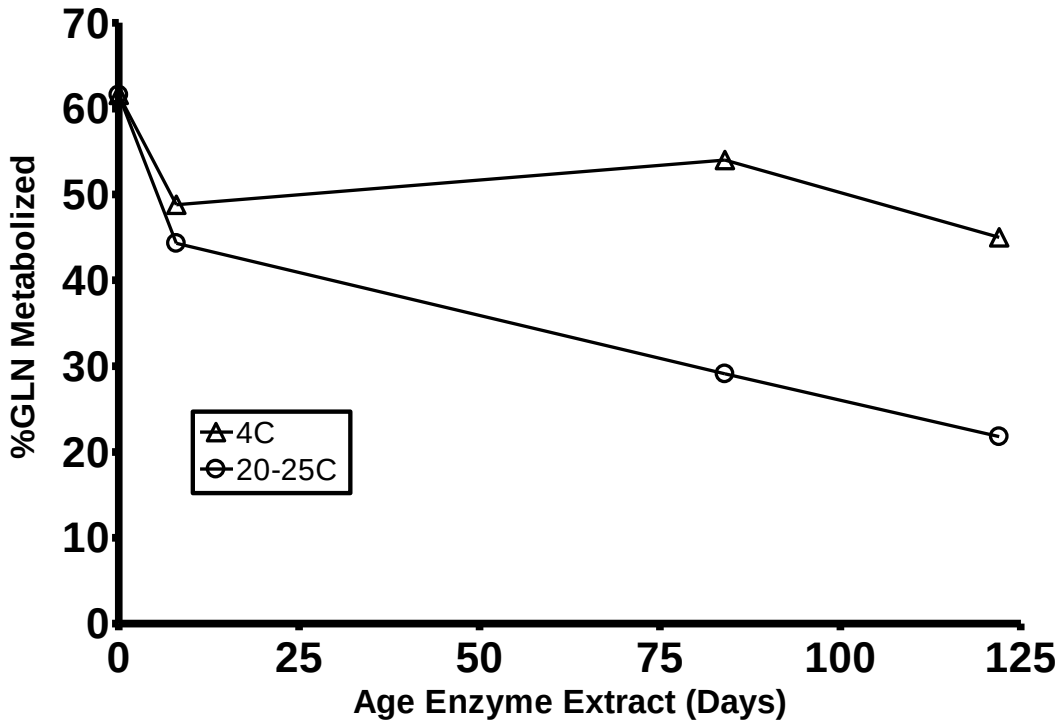

Fig 12. Stability of myrosinase activity in 20% EtOH extract stored at 20-25°C and 4°C. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with water. The 23-hour fermentation used a water matrix and 0.0025 g seed per g MSM.

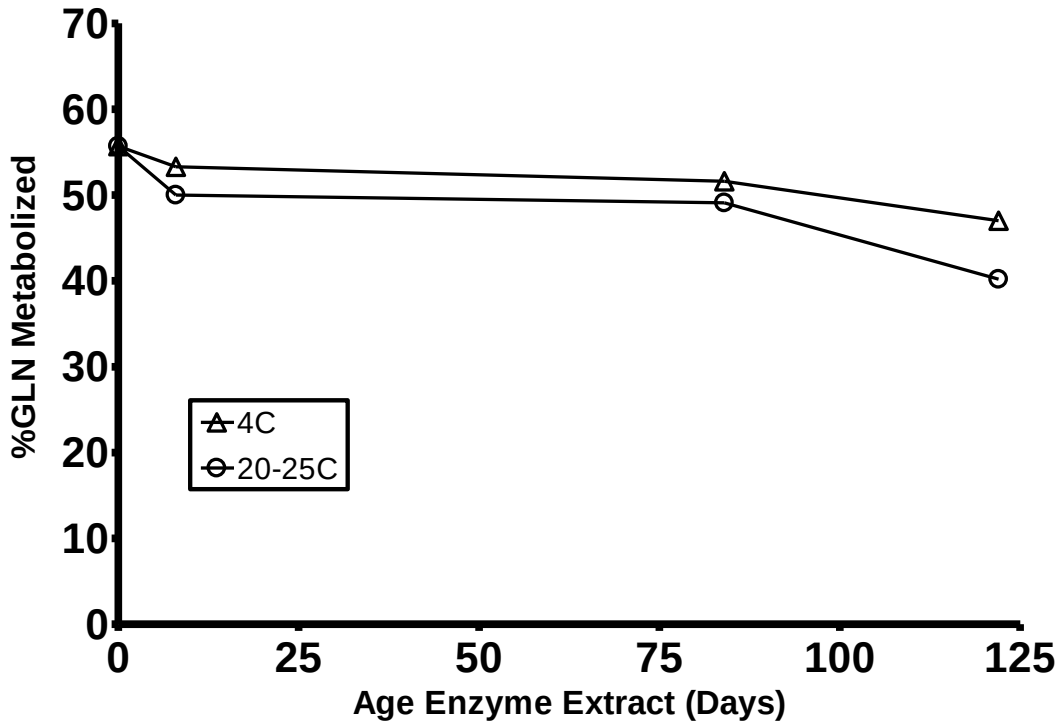

Fig 13. Stability of myrosinase activity in 2% NaCl extract stored at 20-25°C and 4°C. 2% NaCl was used as solvent in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with water. The 23-hour fermentation used a water matrix and 0.0025 g seed per g MSM.

GLN Fermentation

The rate and extent of GLN conversion to bioherbicidal breakdown products during fermentation are dependent upon multiple factors such as MSM extraction, seed extraction, and fermentation conditions. The %EtOH present in the fermentation matrix was investigated as an influencing factor on the conversion of GLN to nitrile and ITC. A 6-day fermentation using 9-96% EtOH in the fermentation matrices revealed that 0-30% EtOH in the fermentation matrix is optimal for the overall conversion of GLN (Fig. 14), 40-60% EtOH in the fermentation matrix is optimal for the conversion of GLN to nitrile

(Fig. 15), and 30-40% EtOH in the fermentation matrix is optimal for the conversion of GLN to ITC (Fig. 16).

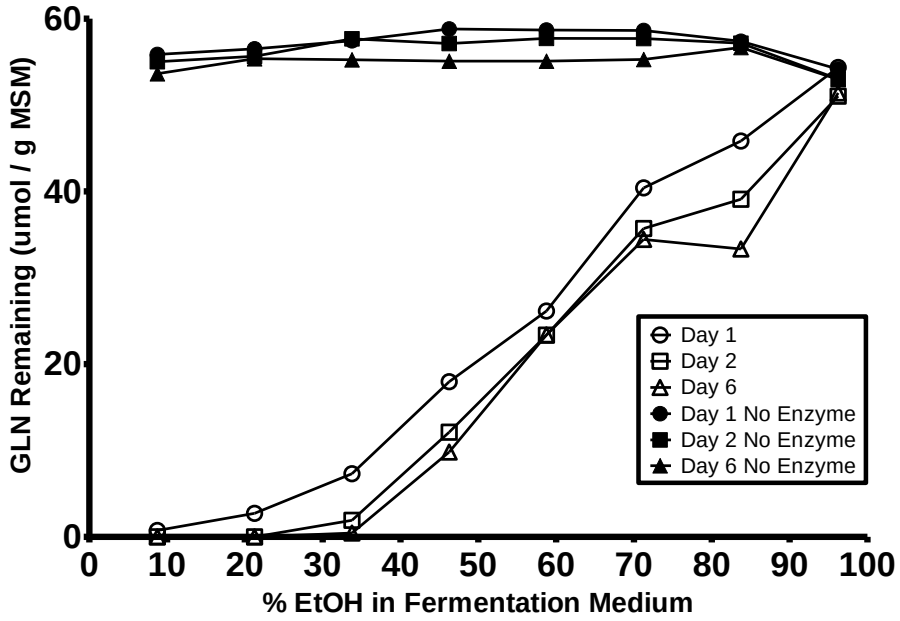

Fig 14. GLN conversion as affected by fermentation matrix. Water was the solvent used in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. Matrices of 0-100% EtOH were used in the 6-day fermentation.

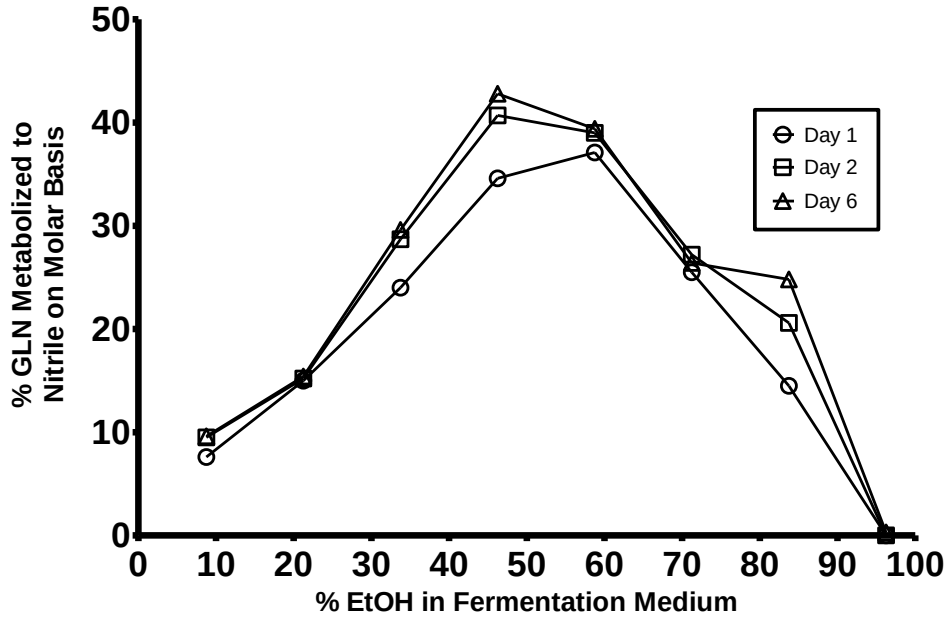

Fig 15. Nitrile production as affected by fermentation matrix. Water was used as solvent in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. Matrices of 0-100% EtOH were used in the 6-day fermentation.

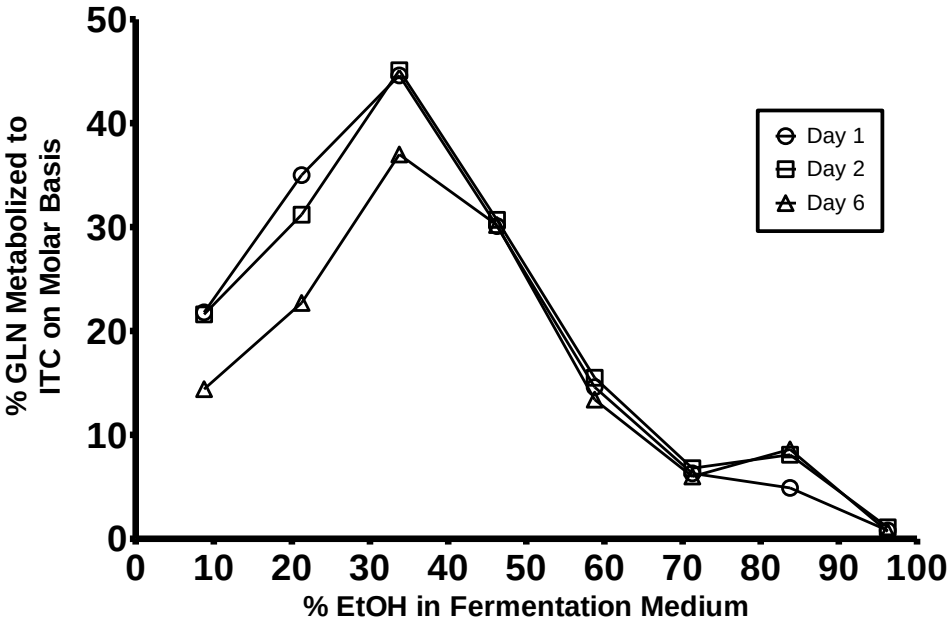

Fig 16. ITC production as affected by fermentation matrix. Water was used as solvent in the 2-hour extraction of myrosinase from seed. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. Matrices of 0-100% EtOH were used in the 6-day fermentation.

It is important to note that although optimal GLN conversion occurs at 0-30% EtOH, that is also the range within these conditions wherein the most GLN is “lost” (metabolized but not accounted for in nitrile and ITC production). The data also shows that both GLN conversion and nitrile production values increase between 1 and 6 days, yet ITC production values decrease over that amount of time. This is not necessarily due to quantitation error or a lack of ITC production from GLN during this time. The cause of this decrease in ITC detection is more likely due to the relative reactivity of ITC as compared to nitrile. GLN is also more stable than ITC, though this is not apparent in these figures since myrosinase is actively converting GLN into nitrile and ITC.

Once 2% NaCl as a seed extraction solvent had been observed as superior to water for the apparent extraction of active myrosinase, the question arose as to whether the presence of NaCl had enhanced enzyme extraction, improved enzyme activity during fermentation, or both. An experiment was conducted comparing two fermentations, one of which received 2% NaCl in the myrosinase extraction solvent, and one of which received an equivalent amount of NaCl in the fermentation matrix after myrosinase had been extracted with water. The results suggested that NaCl-containing solvents extract more myrosinase during seed extraction, yet had no effect or perhaps even a slightly negative effect on GLN conversion when present in the fermentation matrix (Fig. 17).

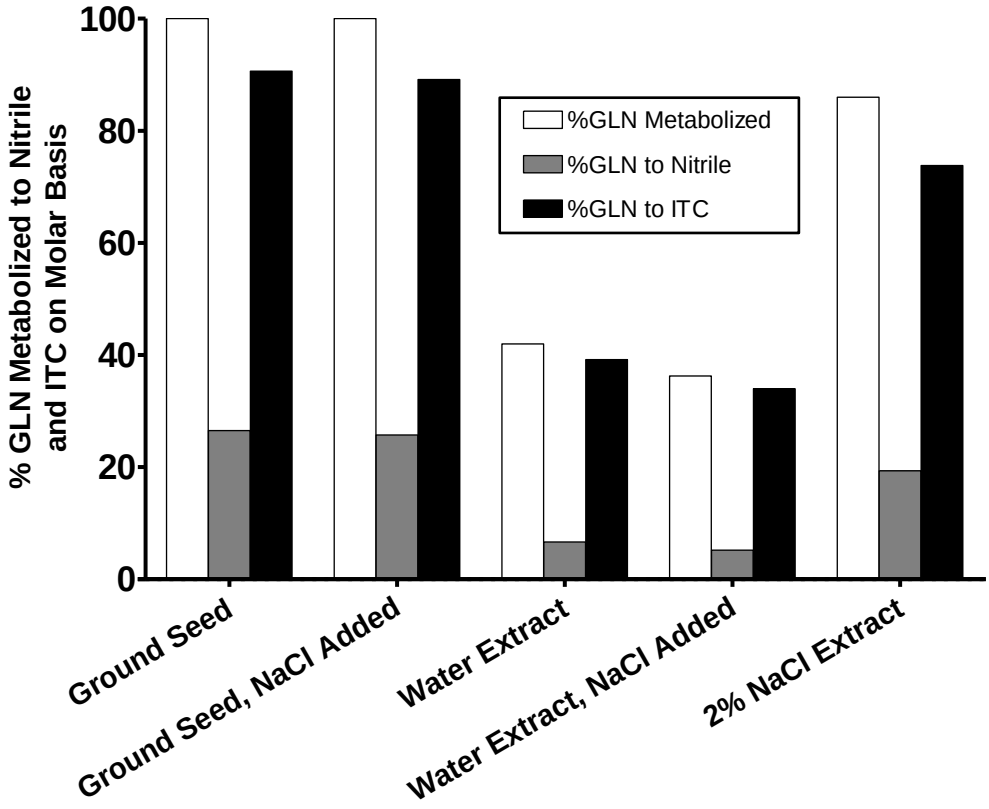

Fig 17. Effect of 2% NaCl on GLN conversion when used in myrosinase extraction vs. merely added to fermentation. 2% NaCl and water were used as solvent in the 2-hour extraction of myrosinase from seed. Ground seed was also used as a myrosinase source. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. The 23-hour fermentation occurred in 35% EtOH, with or without the addition of NaCl.

After multiple experiments were conducted that used a GLN extraction solvent common to all samples, while observing variation in the fermentation matrices, the opposite was explored. Findings demonstrated that GLN fermentation, wherein %EtOH in the fermentation matrix was equal for all samples, created differing ratios of GLN metabolites dependent upon the %EtOH in the GLN extraction solvent. In a 35% EtOH fermentation matrix, increasing %EtOH in the extraction solvent shunts GLN conversion away from ITC production, towards nitrile production (Fig. 18, 19, 20).

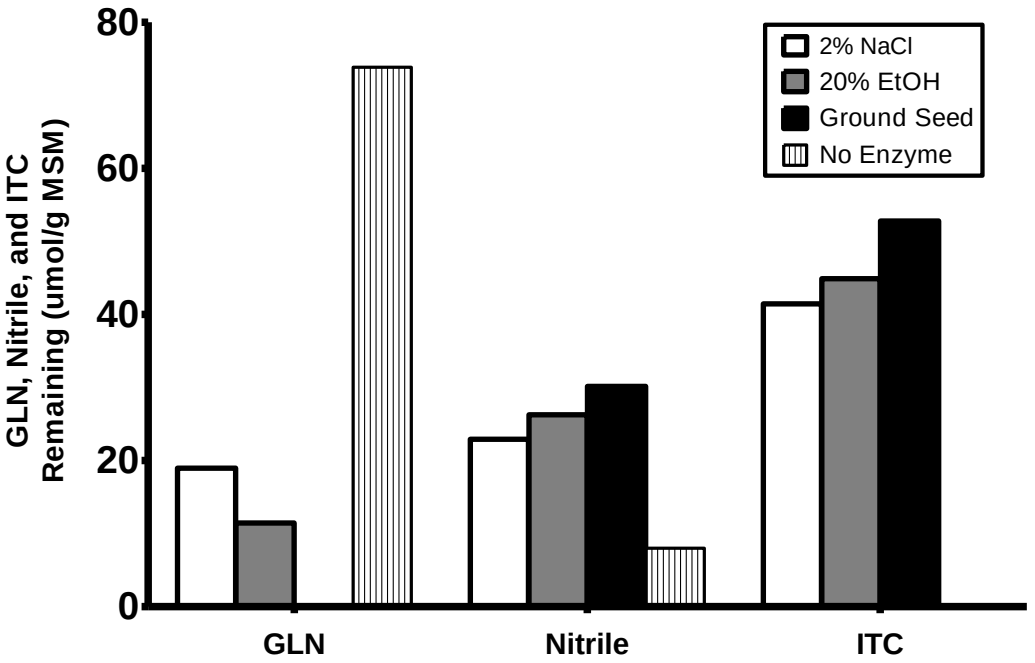

Fig 18. Effect of MSM extraction solvent and myrosinase source on GLN conversion. 2% NaCl and 20% EtOH were used as solvent in the 2-hour extraction of myrosinase from seed. Ground seed was also used as a myrosinase source. The substrate solution containing GLN was obtained by extraction for 2 hours with water. The 23-hour fermentation was performed in 35% EtOH.

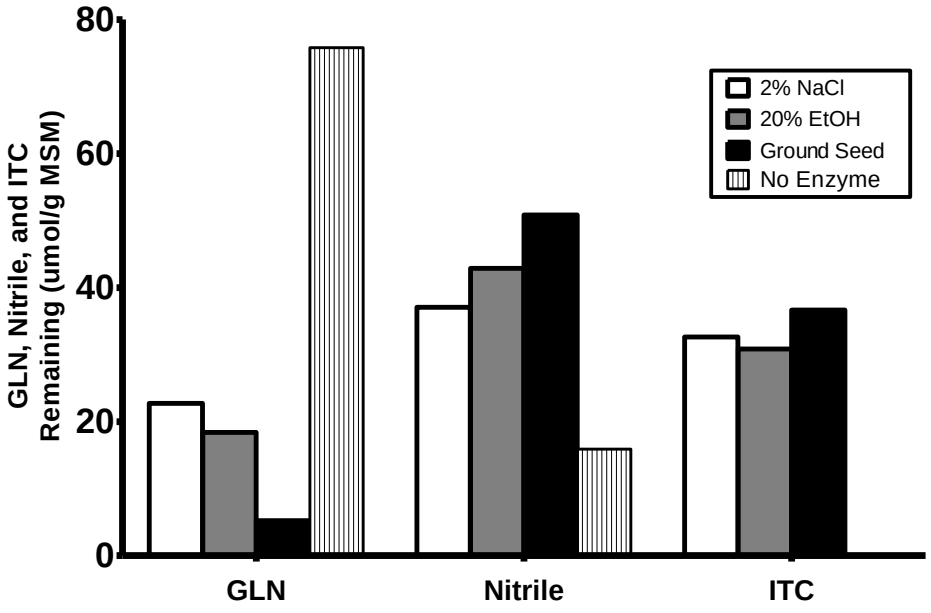

Fig 19. Effect of MSM extraction solvent and myrosinase source on GLN conversion. 2% NaCl and 20% EtOH were used as solvent in the 2-hour extraction of myrosinase from seed. Ground seed was also used as a myrosinase source. The substrate solution containing GLN was obtained by extraction for 2 hours with 35% EtOH. The 23-hour fermentation was performed in 35% EtOH.

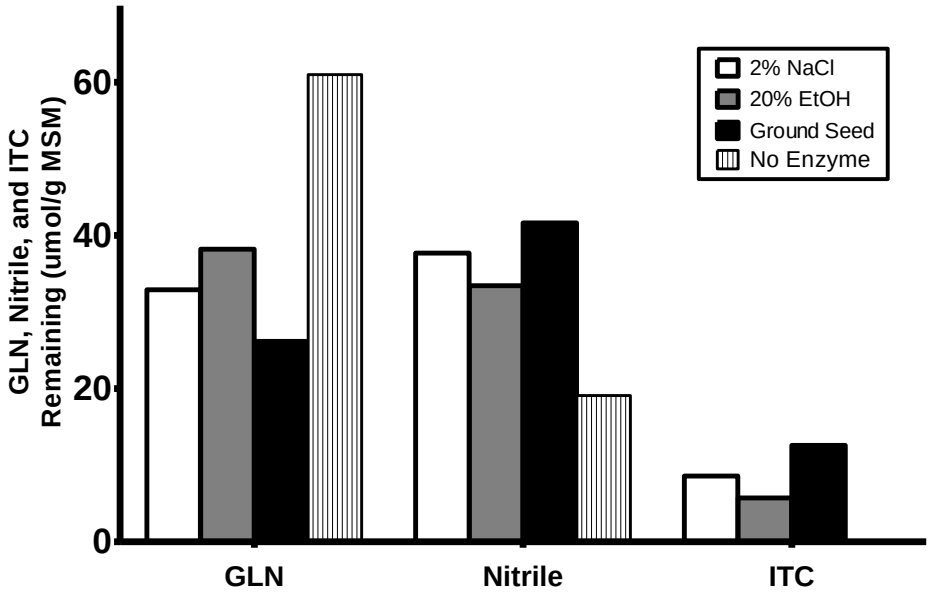

Fig 20. Effect of MSM extraction solvent and myrosinase source on GLN conversion. 2% NaCl and 20% EtOH were used as solvent in the 2-hour extraction of myrosinase from seed. Ground seed was also used as a myrosinase source. The substrate solution containing GLN was obtained by extraction for 2 hours with 70% EtOH. The 23-hour fermentation was performed in 35% EtOH.

This novel finding suggests that something may be extracted from the MSM besides GLN and nitrile that is causing the observed change in products created. It is also possible that the higher % EtOH inhibits the “Lossen rearrangement” activity of myrosinase. The unknown factor or factors extracted in differing quantities with variable %EtOH in the GLN extraction solvent have yet to be identified. Logical suspects must originate from MSM and affect fermentation due to presence or absence in the fermentation matrix. Hypothesized culprits include a protein, combination of proteins, a new (not myrosinase) enzyme, multiple new enzymes, inhibition of the “Lossen rearrangement”, or some combination of these factors that are introduced to the fermentation matrix via the MSM extract.

A notable variation is observed between the relative effectiveness of 20% EtOH vs. 2% NaCl as seed extract with increasing %EtOH in the MSM extraction solvent. As more EtOH is added to the MSM extraction solvent, the 2% NaCl seed extract becomes more effective relative to the 20% EtOH seed extract (Fig. 18, 19, 20). The cause of this shift with increasing %EtOH in the MSM extraction solvent is unknown, yet may relate to the suspected presence/absence of fermentation matrix compounds from MSM.

Discovery of a method to almost exclusively convert GLN to ITC was also made during this experiment (Fig. 21).

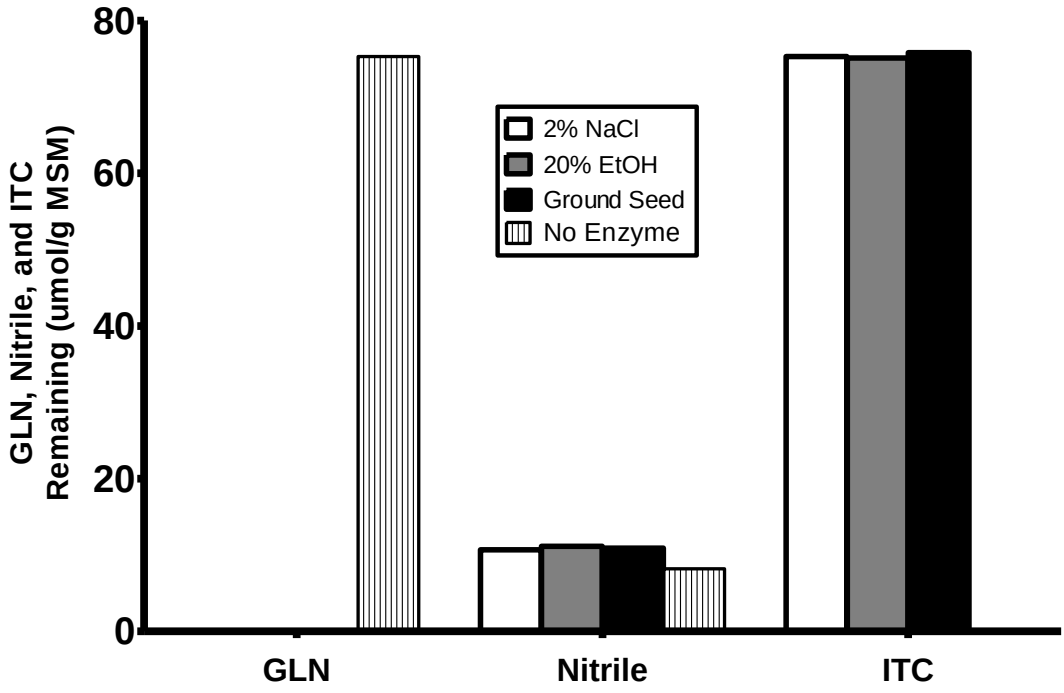

Fig 21. Effect of MSM extraction solvent and myrosinase source on GLN conversion. 2% NaCl and 20% EtOH were used as solvent in the 2-hour extraction of myrosinase from seed. Ground seed was also used as a myrosinase source. The substrate solution containing GLN was obtained by extraction for 2 hours with water. The 23-hour fermentation was performed in water.

Such a successful ITC recovery was achieved using water as solvent in both extraction and fermentation matrices. These results may appear confounding when it is recalled that prior experiments suggested that a fermentation matrix of 0-30% EtOH is the range in which the most GLN is converted but “lost” (not recovered as nitrile or ITC) and that ITC is produced in the greatest quantity between 30-40% EtOH in the fermentation matrix. However, those findings were produced using an MSM extraction solvent of 70% EtOH rather than water as in this experiment. Nearly 100% of GLN present in MSM is extracted, virtually 100% of GLN extracted was converted during fermentation, and almost 100% of that converted GLN was accounted for as ITC recovered. These findings suggest the discovery of a very successful, fast, and cheap method for the recovery of ITC from MSM, although the ITC within this extract has not yet been stabilized by any means.

Findings from the previous experiments (Fig. 18, 19, 20, 21) gave rise to the idea that adjusting the %EtOH used the create the MSM extract would change more factors than just the ratio of nitrile/ITC recovered or the relative effectiveness of EtOH vs. NaCl seed extracts, as observed. The data suggested that all findings up to that point in time were meaningful only when the same MSM extract, seed extract, and fermentation matrix that was employed for any given experiment was used. This inspired the conduction of a “matrix” experiment, plotting GLN converted, nitrile recovered, and ITC recovered as a result of varying %EtOH in the MSM extraction solvent and in the fermentation matrices during the same experiment.

Variation of %EtOH was limited to 35-70% for MSM extraction since extractions above 70% EtOH extracted GLN less effectively, and extractions below 35% EtOH retained unnecessary amounts of protein without additional GLN yield (Fig. 3, 4). The range 35-60% EtOH for GLN fermentation matrices was chosen, as fermentation above 60% converted an insufficient amount of GLN, and fermentation below 35% EtOH resulted in the most GLN converted but “lost” (Fig. 14, 15, 16). Within these ranges, 35% EtOH in the MSM extraction solvent and 35% EtOH in the fermentation matrix were the most effective for overall conversion of GLN (82%), conversion of GLN to nitrile (42%), and conversion of GLN to ITC (45%) (Fig. 22, 23, 24). The greatest ratio of nitrile/ITC (5.6) recovered (Fig. 25) was found using 70% EtOH in the MSM extract and 55% EtOH in the fermentation matrix. When considering total nitrile recovery (extracted from MSM and converted from extracted GLN) 45-55% EtOH in the MSM extraction solvent and 35% EtOH in the fermentation matrix appeared to be optimum, with 47 umol nitrile recovered per gram MSM extracted (Fig. 26).

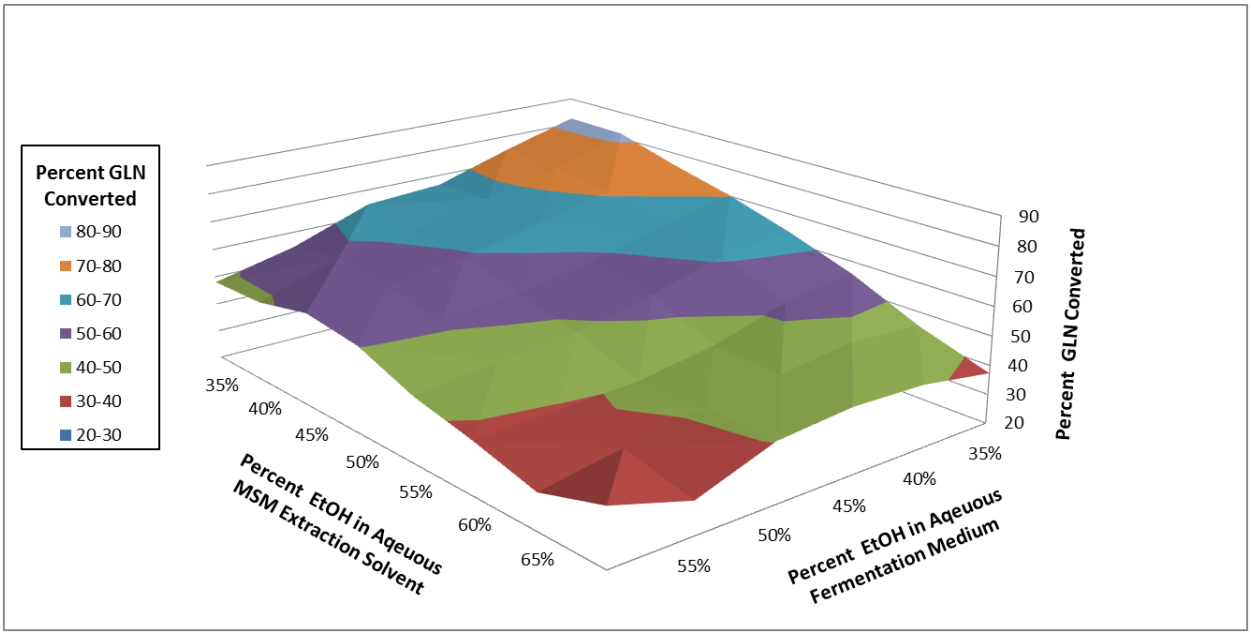

Fig 22. Effect of MSM extraction solvent and GLN fermentation matrix on overall GLN conversion. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The fermentation lasted 23 hours.

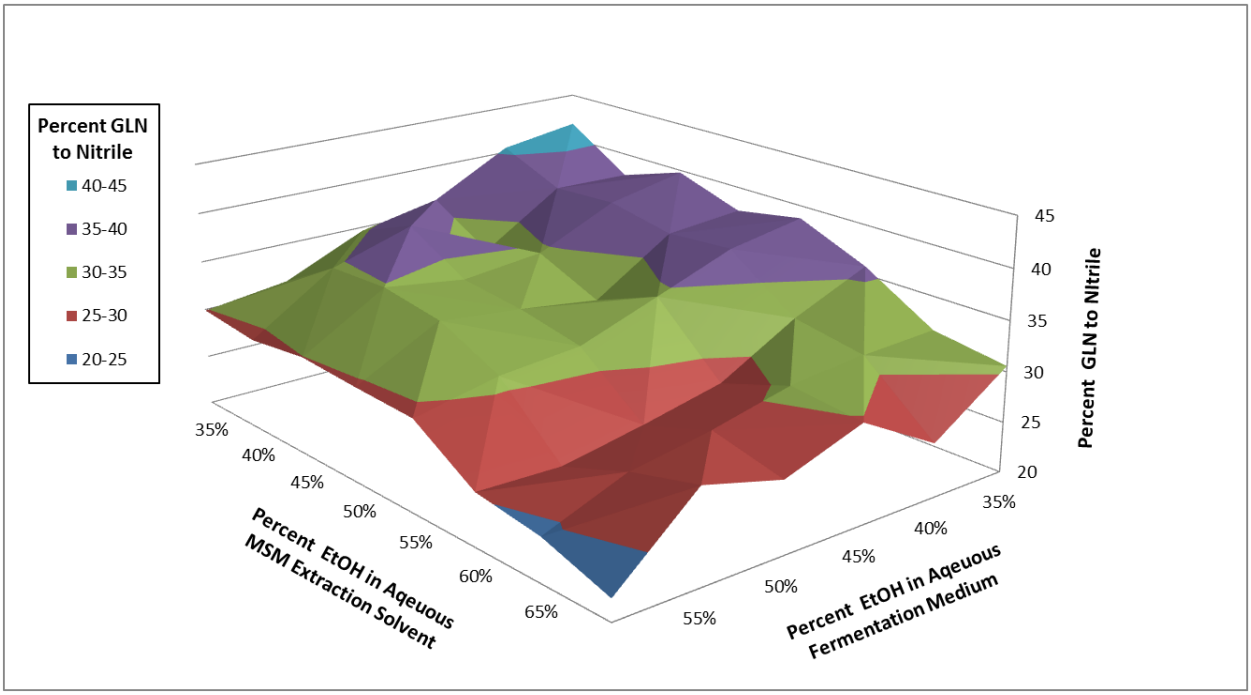

Fig 23. Effect of MSM extraction solvent and GLN fermentation matrix on GLN conversion to nitrile. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The fermentation lasted 23 hours.

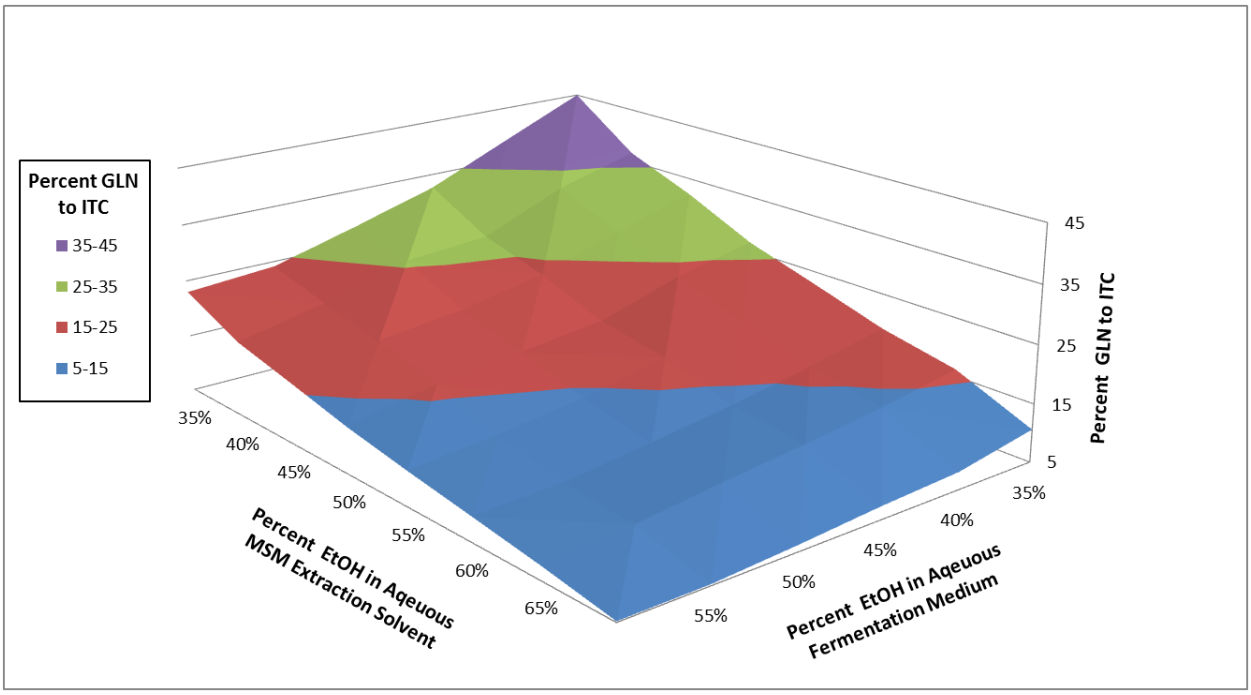

Fig 24. Effect of MSM extraction solvent and GLN fermentation matrix on GLN conversion to ITC. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The fermentation lasted 23 hours.

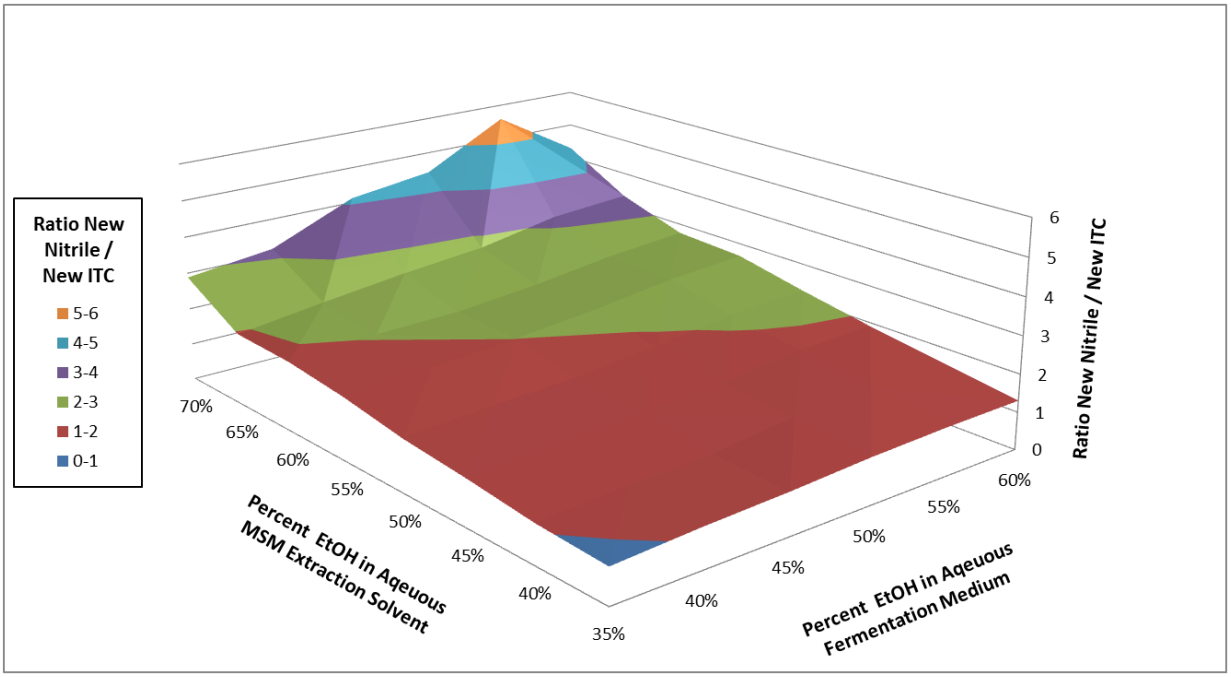

Fig 25. Effect of MSM extraction solvent and GLN fermentation matrix on ratio of nitrile/ITC converted from GLN. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The fermentation lasted 23 hours.

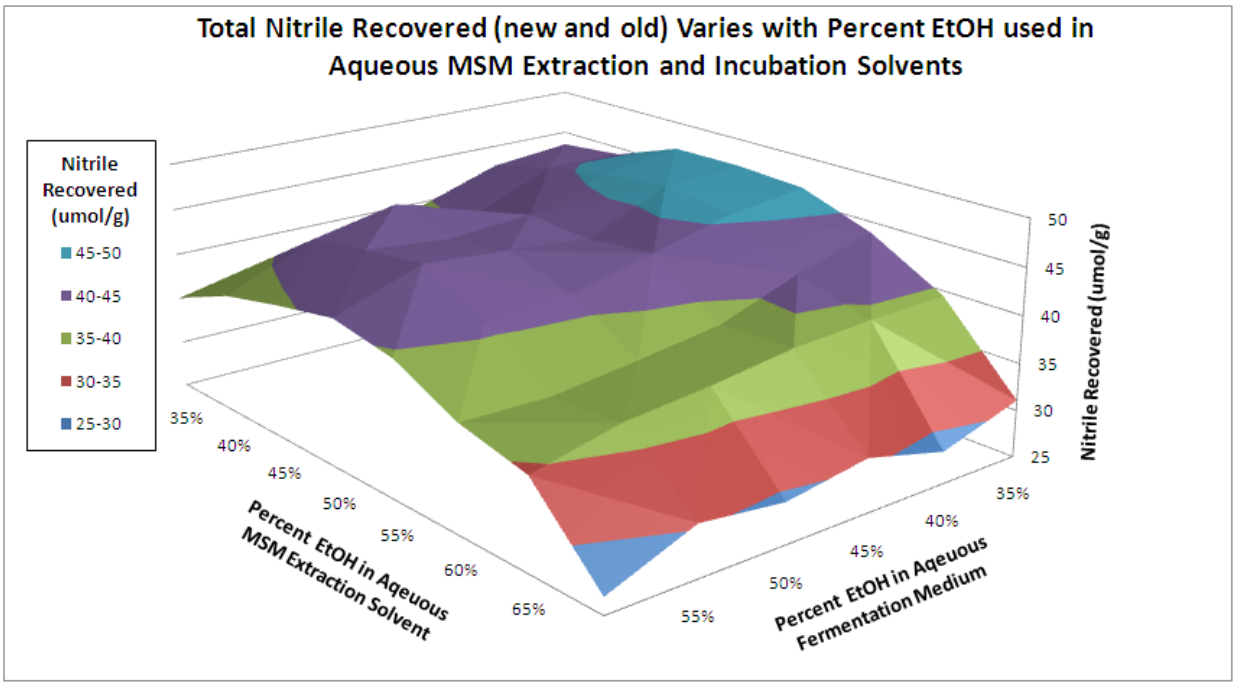

Fig 26. Effect of MSM extraction solvent and GLN fermentation matrix on total nitrile production. 20% EtOH was used as solvent in the 2-hour extraction of myrosinase from seed. The fermentation lasted 23 hours.

These “matrix” findings confirmed that GLN conversion to bioherbicides is the product of both MSM extraction solvents and GLN fermentation matrices. The ratio of GBPs created was found to be dependent on the solvents used for extraction of MSM, for extraction of seed, and as fermentation matrices. “Matrix” trends were consistent with prior experiments concerning GLN extraction and fermentation conditions as they affect nitrile and ITC production. This research has shown the feasibility of combining liquid MSM extracts with liquid seed extracts for the purpose of catalyzing the fermentation of GLN to form the bioherbicidal GBPs nitrile and ITC. The activity of enzyme extract over time was assessed and found to be stable under the proper storage conditions. The developed method is promising for the production of economical bioherbicides from the abundant by-product MSM.

Acknowledgements

I owe a tremendous debt of gratitude to several faculty members at Oregon State University for their continued guidance and support over the past years. I must express great thanks to my primary mentor, Dr. Fred Stevens, for accepting me into his laboratory as an undergraduate intern in pursuit of a degree in Bioresource Research (BRR), and allowing me the use of all of the facilities and instrumentation at his disposal. The upper-level chemistry courses in which he encouraged me to enroll such as Quantitative Analysis and Analytical Chemistry have provided me with some of the most salable and transferable skills on my resumé, as did the summer research position that he was kind enough to offer me between academic terms. Dr. Stevens' mentorship and patience were invaluable aids in the completion of this research project and undergraduate degree.

An enormous thank you to my secondary mentor, Dr. Ralph Reed, for the countless hours spent teaching me the good laboratory practices and investigative techniques necessary to properly conduct research as well as the theoretical significance behind these methods. Dr. Reed's uncommonly kind friendship and fantastic mentorship throughout this investigation have provided me with a greater wealth of knowledge and practical skill than any typically impersonal university lecture series I have experienced, for which I will always be grateful.

Thank you to the advisor of the BRR major, Wanda Crannell, for the unending resources that she provides. Whether I was currently enrolled in one of her courses or

not, Wanda Crannell has constantly provided me with information on graduate programs, career opportunities, scholarship information, and other valuable offers of which I would otherwise have been completely unaware. She is the type of instructor that remembers students by name, and goes even further to get to know them on a more personal level. On multiple occasions Wanda Crannell has engaged me in conversation about my future after graduating from Oregon State University. She later recalls these conversations, going above and beyond what I have observed or would expect of any other academic advisor to provide encouraging resources and advice towards the completion of these goals.

I would like to offer a final thank you to Dr. Kate Field, BRR program director, for her help with the completion and revision of my undergraduate thesis. Her positive demeanor and willingness to teach was a great asset throughout the process. It is thanks in great part to these educators that this comprehensive experience is available. I hope that the BRR major continues to grow, to aid in the academic and vocational development of many more undergraduate students such as myself in the years to come.

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