Short Communication. Bio-oil extraction of *Jatropha curcas* with ionic liquid co-solvent: Fate of Biomass Protein. Michael J. Cooney[✉], Godwin Severa[✉], and Melisa Edwards. Hawaii Natural Energy Institute, University of Hawaii at Manoa, 1680 East West Road, POST 109, Honolulu, HI 96822.

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ABSTRACT

The fate of oil-seed biomass protein has been tracked through all steps of a multi-phase extraction process using an ionic liquid based co-solvent system previously demonstrated to extract bio-oil and phorbol esters and to recover fermentable sugars from *Jatropha* oil seed. These analyses, however, did not address the fate of biomass protein. This work demonstrated that the majority of protein (~ 86%) tracked with the biomass with the balance lost to co-solvent (~ 12%) and methanol (~ 2%) washes. A significant portion of the ionic liquid remained with the treated biomass and required aggressive methanol washes to recover. A system analysis showed a net-positive energy balance and thus the potential of this system to produce both bio-oil and protein-rich toxin-free biomass. While these results further support *Jatropha* as an oil seed crop, the additional costs of solvent recovery will need to be addressed if commercialization is to be realized.

Keywords: bio-oil bearing biomass, *Jatropha*, ionic liquid co-solvents, protein extraction.

1. INTRODUCTION

Bio-oil from non-food bio-oil seeds (second generation 2G) can overcome the shortcomings of first generation (1G) biodiesels (which are derived from edible sources) in addressing social, economic and environmental challenges without hampering food cost and creating pressure on land use (Azad et al. 2015). Commercialization, however, is challenged owing to a lack of sophisticated processing equipment, investment per unit fuel production and large scale facilities (Azad et al. 2015) as well as the marginal value of the bio-oil product (Klein-Marcuschamer et al. 2012) and high process costs (Sanjid et al. 2013). As with microalgae (Martin 2016), improving commercial viability will require energy efficiency and the additional recovery of value added byproducts such as fermentable sugars, chemicals, and protein as well as avoiding competition for land, water and other resources that would otherwise be dedicated to food production (Mofijur et al. 2015). *Jatropha curcas* is one such popular non-food oil-seed crop promoted as a renewable feedstock for bio-oil production (Achten et al. 2008, dos Santos et al. 2015). In addition to having a comparatively positive environmental life cycle (Ajayebi et al. 2013), being well adapted to marginal areas

with poor soils and low rainfall, resistant to diseases and herbivores, and possessing bio-oil yields as high as 60% (Severa et al. 2013, Najjar et al. 2014, Severa et al. 2014), *Jatropha* seed kernels also contain significant amounts of carbohydrates and proteins (Severa et al. 2013), both of which are potentially valuable in animal feeds. If the protein could be recovered within a toxin free delipified biomass, the potential to grow *Jatropha* as an energy crop on marginal lands would be greatly improved.

The extraction of bio-oil from *Jatropha* biomass with alcohols has been shown to lead to a higher efficiency of protein recovery from the delipified biomass as compared to conventional mechanical de-oiled biomass (Martinez-Maqueda et al. 2013). Alcohols have been reported to enhance protein solubility in other solvents, by increasing protein accessibility to the solvent molecules. Alcohols like methanol are highly polar small molecules with significant hydrogen bond capability which can effectively penetrate and disrupt biomass matrices (Yang et al. 2015). Methanol containing ionic liquid co-solvents were demonstrated to extract multiple components from bio-oil bearing biomass (Young et al. 2009). In one specific application the simultaneous extraction of bio-oil and phorbol esters from *Jatropha* oil-seed was successfully achieved, with the bio-oil partitioned to a separate immiscible phase and over 98% of phorbol esters extracted into the co-solvent (Severa et al. 2013). The fate of the protein nitrogen throughout the multi-phase extraction process, however, was not quantified.

An assessment of the co-solvent system's ability to support a *Jatropha* oil seed industry must track the fate of the protein through all stages of the extraction process. Without this data a useful analysis is limited. This study quantitatively tracked the biomass protein through all extraction steps and reported on final protein concentrations in all terminal phases of the extraction process. A relatively extensive energy balance has also been executed at scale. Results indicate that the hydrophilic ionic liquid 1-ethyl-3-methylimidazolium acetate - methanol co-solvent extracted very little protein, leaving the majority with the biomass despite multiple solvent washing steps used to recover residual ionic liquid from the biomass.

More, the system showed a net-positive energy balance although solvent recovery costs were not included.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Jatropha seeds (19.5% protein, 29.7% lipid, 47.8% carbohydrate, on a w/w basis) were obtained from University of Hawaii, Department of Tropical Plant and Soil Science. 1-ethyl-3-methylimidazolium acetate ([C₂mim][Ac], 16.5% N on a w/w basis) 90% (Aldrich), methanol (Fisher scientific), hexane (Fisher scientific), modified Lowry protein assay kit (Thermo scientific), Bradford protein assay kit (Thermo scientific) and total Kjeldahl nitrogen reagent set (#8075, Hach Company) were used as received. Sulfuric

acid (Fisher scientific), and 30% hydrogen peroxide (Fisher scientific) were used as received. Water (resistivity 17.5 MΩ cm) was used to make all aqueous solutions (Barnstead E-pure water purification system). TKN analysis applied to [C₂mim][Ac] returned a value of 16.34 ± 0.8 % N (w/w basis), verifying equivalency to the empirical estimation of nitrogen (16.5% w/w) in [C₂mim][Ac].

2.2. Co-solvent treatment pathway

The treatment of Jatropha biomass was executed across multiple steps (Figure 1). Initially, 0.8 (or 1.6) grams of ground whole Jatropha seed biomass (1, Figure 1) was transferred to pre-weighed glass tubes filled with 3.2 (or 6.4) grams of 70:30 w/w mixture of 1-ethyl-3-methylimidazolium acetate ([C₂mim][Ac]) to methanol (CH₃OH) co-solvent (2, Figure 1) to a final solids loading of approximately 20% (w/w). The mixture was then heated under seal to 120°C for five hours with mild mixing achieved using a magnetic stir bar (3, Figure 1).

Upon completion of the extraction process (3, Figure 1), the mixture was transferred to a plastic 50 ml centrifuge tube using 25 ml of methanol rinse (4, Figure 1) after which the entire mixture was centrifuged at 6200 rpm for 30 minutes to produce three phases: a bottom delipidified biomass phase, a middle co-solvent phase, and a top lipid phase (5, Figure 1). After centrifugation, both the top bio-oil (lipid) rich layer and middle co-solvent layer were transferred by pipette to another pre-weighted glass tube (6, Figure 1), leaving behind the bottom delipidified biomass (delipidified seedcake) phase in the centrifuge tube (13, Figure 1).

The combined top bio-oil rich and middle co-solvent phases (6, Figure 1) were then washed (7, Figure 1) three times with 10 ml of hexane (8, Figure 1). After each wash step the hexane was recovered by pipette suction, pooled together in a separate pre-weighed glass tube (9, Figure 1), and heated under vacuum (10, Figure 1) using a rotary evaporator (Buchi, model R-210) to produce a concentrated bio-oil residue (11, Figure 1). This residue was then weighed to determine the gravimetric bio-oil yield (g g⁻¹) before being stored at 4°C until analyzed for protein using TKN analysis. The TKN analysis was used (as opposed to Commassie-Bradford assay) because it was assumed that the co-solvent would not absorb into the hydrophobic bio-oil (thus ensuring all measured nitrogen came from protein and not co-solvent) and because the TKN assay was deemed a more accurate assessment of protein nitrogen contained within a bio-oil matrix. The remaining middle co-solvent phase (12, Figure 1) was then stored at 4°C until analyzed using both the Commassie-Bradford and TKN assays. Both assays were required to distinguish between the relative contribution of nitrogen coming from protein and the imidazolium ring of the ionic liquid.

The delipidified seedcake (13, Figure 1) was re-suspended (14, Figure 1) three times with 10 ml of methanol (15, Figure 1). Each seedcake-methanol resuspension (16, Figure 1) was centrifuged at

6,200 rpm for 30 minutes (17, Figure 1) to produce two layers: a top methanol wash (18, Figure 1) and a bottom methanol washed seedcake (19, Figure 1). The methanol washes were pooled into a pre-weighed tube (18, Figure 1) and then further washed (20, Figure 1) five times with 5ml of hexane (21, Figure 1). Each hexane layer was recovered and pooled within a pre-weighed tube (23, Figure 1) and then concentrated under rotary evaporation (24, Figure 1) to produce a concentrated bio-oil (25, Figure 1). The amount of additional bio-oil residue recovered from the methanol wash (18, Figure 1) was determined by difference. The bio-oil residue (25, Figure 1) was stored at 4°C until analyzed for protein nitrogen using the TKN analysis because it was assumed that the hydrophilic co-solvent did not absorb into the hydrophobic bio-oil (thus eliminating any contribution of nitrogen from the co-solvent) and because the TKN assay was deemed a more accurate assessment of nitrogen contained within a bio-oil matrix. The washed methanol (22, Figure 1) was stored at 4°C until analyzed for protein using the Commassie-Bradford assay and total nitrogen using the TKN assay.

The methanol washed seedcake (19, Figure 1) was washed (26, Figure 1) an additional five times with 5ml of hexane (27, Figure 1). All hexane washes were pooled into a pre-weighed tube (29, Figure 1). heated under vacuum (30, Figure 1) using a rotary evaporator (Buchi, model R-210). The concentrated bio-oil residue was weighed and the amount of bio-oil recovered was determined by difference. The recovered bio-oil (31, Figure 1) was then stored at 4°C until analyzed for total nitrogen using TKN analysis. As with the other bio-oil residues (11, Figure 1 and 25, Figure 1) the protein nitrogen was estimated by TKN analysis on the assumption that the hydrophilic co-solvent did not absorb into the hydrophobic bio-oil (thus eliminating any contribution of nitrogen from the co-solvent) and because the TKN assay was deemed a more accurate assessment of nitrogen contained within a bio-oil matrix. The remaining seedcake (28, Figure 1) was then transferred from its centrifuge tube into a pre-weighed aluminum boat and dried at 80°C and stored at 4°C until analyzed for total protein nitrogen using the TKN analysis.

2.3. Assays

2.3.1 Total Kjeldahl Nitrogen. Total nitrogen (w/w) measurements were performed using the Kjeldahl method (Hach Company, Model 23130-20). Bio-oil samples (11, 25, and 31, Figure 1) were analyzed individually while co-solvent (12, Figure 1), washed methanol (22, Figure 1), and seedcake (28, Figure 1) samples were analyzed in triplicate. Samples (~0.1 gram) were transferred into 100 mL digesdahl digestion flasks to which 4 mL of sulfuric acid was added (specific gravity of 1.84) and the digestion operated as per directions (Hach Company, Digesdahl Digestion Apparatus Models 23130-20, -21 Instrument Manual, Digestion Procedures, pp 27-79, Edition 8, revised 1999). The amount of total nitrogen recovered was then

quantified by application of the modified Nessler method (Hach kit #2495300). For samples where no nitrogen was assumed derived from the digestion of ionic liquid, the total nitrogen was assumed to derive from protein and its amount estimated from the total nitrogen value by multiplying the TKN value by 6.25.

2.3.2 Protein Nitrogen. The Coomassie Bradford spectrophotometric assay (Thermo Scientific.

Instructions: Coomassie (Bradford) Protein Assay Kit 23200. Thermo Fisher Scientific Inc. 2014) was used to directly measure protein in co-solvent (12, Figure 1), washed methanol (22, Figure 1), and seedcake (28, Figure 1) samples. To avoid methanol interference, both the recovered co-solvent and methanol wash phases were diluted with pure water at a 1:1 ratio. Samples (30 μ l) of either standard or unknown (recovered co-solvent and methanol wash) were pipetted into appropriately labeled test tubes. 1.5 ml of Blue Coomassie Reagent was then added to each tube, mixed, and then incubated for 10 min at room temperature (RT). The absorbance of all samples was measured three times at 595 nm and after the instrument was first zeroed to deionized water. To average out error measurements were performed in triplicate. Standard curves were prepared using bovine serum albumin (BSA) as the external standard.

3. RESULTS AND DISCUSSION

3.1. Bio-oil recovery

Contrary to previous studies (Severa et al. 2013, Severa et al. 2013, Severa et al. 2014), only half (~55% w/w, Table 1) of the total lipid available in the *Jatropha* biomass was extracted during the initial extraction step (5, Figure 1). This discrepancy is attributed to a modification of the extraction process to accommodate the focus on tracking biomass protein. Specifically, this experiment used 25 ml instead of 5 ml of methanol in the transfer of extracted material (4, Figure 1) to the centrifuge tube (5, Figure 1). This likely caused a greater portion of the oil to remain with the bottom biomass phase as lipids are denser than methanol. The majority of this bio-oil was then recovered by a combination of washes (19, 27 Figure 1) of the delipidified biomass. In a typical application of this solvent system, however, this modification would not be used and thus full recovery of the lipid is expected as reported elsewhere (Severa et al. 2013, Severa et al. 2013, Severa et al. 2014).

3.2. Protein extraction and distribution

The tunable physicochemical properties of ionic liquids have encouraged research regarding their effectiveness as solvents for protein extraction (Alvarez-Guerra et al. 2012). Martínez-Aragon et al. (2009) compared the extraction of protein into hydrophobic ionic liquids relative to aqueous and hydrophilic (i.e. n-

butanol) and hydrophobic organic (i.e. hexane) solvents (Martínez-Aragón et al. 2009). The authors reported protein retention within hydrophobic ionic liquids was equal to or even better than conventional solvents (Martínez-Aragón et al. 2009). Similar findings were also reported by Lau et al. who reported the solubility of proteins to decrease with alkyl chain length on the imidazolium ionic liquid cation. Contradicting results, however, reported hydrophilic ionic liquids to better stabilize proteins as compared to hydrophobic ionic liquids (Solhtalab et al. 2015). Ionic liquids containing anions with low hydrogen bonding ability were also reported to stabilize proteins (Zhao et al. 2009). These contradictions can be attributed to multiple competing factors (IL alkyl chain length, IL polarity, type of IL anion, IL hydrophobicity and IL viscosity) which impact protein stability and hence their solubility in ionic liquid solvents (Cooney et al. 2016). Protein extraction is also dependent upon the physiochemical properties of the matrix from which the protein is extracted, e.g. protein extraction from biomass is different than extraction of protein from solutions. As such, most systems need to be evaluated on a case by case basis.

The final distribution of protein across all steps is presented in Table 1. A small yet significant amount of protein (~12 % w/w) extracted into the hydrophilic 1-ethyl-3-methylimidazolium acetate – methanol co-solvent. Although the general surface hydrophobicity of proteins promotes aggregation and loss of solubility in hydrophilic solvents (Wagner et al. 2000), the 1-ethyl-3-methylimidazolium acetate - methanol co-solvent possesses an uneven heterogeneous distribution of charge and hydrophobic vs. hydrophilic regions on a molecular level (Cooney et al. 2016). These unevenly distributed pockets of hydrophilic regions could interact with the protein's charged polar surface residues to support a small but significant amount of protein solubility.

Proteins tend to be unstable in pure alcohol solvents and thus their solubility is markedly lower (Pace et al. 2004). Consequently, a small amount of protein was recovered in the methanol wash phase (~1.3 % w/w) despite literature reports that alcohols enhance protein accessibility by disrupting biomass matrices. As expected, negligible amounts of protein (~0.004% w/w) were recovered in the highly hydrophobic bio-oil. The majority (~86% w/w) of protein remained with the delipified biomass (28, Figure 1) likely because the elevated temperature caused the protein to coagulate into a form that more tightly bound to the delipified biomass. Despite the efficacy of the 1-ethyl-3-methylimidazolium acetate – methanol co-solvent to penetrate and treat the lignocellulosic biomass, a process that has been shown by the authors to effectively liberate bio-oil from cell tissues (Severa et al. 2013, Severa et al. 2013, Severa et al. 2014), the majority of *Jatropha* proteins were not absorbed into the hydrophilic IL-methanol co-solvent presumably due to the lower solubility of the *Jatropha* proteins in the hydrophilic ionic liquid co-solvent.

3.3. Systems Analysis

The results above suggest the production of a bio-oil and toxin-free protein-rich biomass is a viable process. To assess **its commercial feasibility**, a detailed pathway to process 250 tons of *Jatropha* oil-seed biomass per day into bio-oil and a delipified toxin-free protein-rich biomass has been **examined** (Figure 2). **This value was chosen because at this rate (i.e. 250 tons/day) processes that use solvent extraction become more economical than those that employ mechanical extraction.** The analysis begins with a mass balance executed over an abridged version of this pathway (Figure 3) where whole *Jatropha* seed flakes are extracted using the ionic liquid co-solvent to produce a top bio-oil phase, a middle co-solvent phase, and a bottom digested biomass phase. The bio-oil is then separated while the co-solvent and biomass are further treated to produce an enriched phorbol ester product, a delipified biomass and a recycled co-solvent. **The ability of bio-oil to separate into its own separate top phase ensures its easy separation from the delipified biomass using low energy unit operations such as decantation.** The delipified biomass is **then** washed and dried to produce a final lipid-free toxin-free protein-rich biomass. Results show that 100 pounds of *Jatropha* whole seed yield approximately 32 pounds of bio-oil, 0.5 lbs. of phorbol ester, and 67.5 pounds of delipified biomass meal, assuming complete recovery of the biomass components (Figure 3).

An energy analysis was then performed on **the** more detailed system description (Figure 2). The assumptions made are presented in Table 2 while energy calculations across unit operations are presented in Table 3. Energy loads for specific unit operations were taken from literature, industry literature, or direct conversations with vendors. *Jatropha* whole seed is first flaked and then processed to the final products (bio-oil, phorbol ester and delipified biomass meal). The energy balance, however, assumes 250 work days per year. The high caloric value of the products, bio oil (17,000 Btu/lb.) and delipified meal (7,800 Btu/lb.) yields a positive energy balance despite energy consumed during flaking, centrifugation, heating co-solvent during extraction, desolvantizing and biomass washing. A surprisingly large amount (~ 31% w/w) of the ionic liquid remained with the delipified biomass (28, Figure 1) and a smaller but significant amount (~ 9% w/w) also lost to the methanol wash (22, Figure 1). Both results are attributed to the charged nature of ionic liquids which would be expected to form strong bonds with cell tissues. Consequently the delipified biomass must be aggressively washed to recover ionic liquid co-solvent prior to its use as an animal feed supplement. The high cost of ionic liquids also impose the need for solvent recovery from all phases of biomass.

4. CONCLUSIONS

The optimal use of this extraction system is to produce a bio-oil and toxin free biomass product. Specifically, a majority (~ 86%) of the *Jatropha* protein remained with the biomass even after all washing steps. A significant portion of the IL, however, remained with the biomass and required an aggressive wash protocol to recover. In industry these washing steps will add significant costs. Although the phorbol esters, if recovered, could be marketed as a natural pesticide (to offset costs) this market is not well developed. While the energy balance and existing market for protein rich animal feed bodes well for *Jatropha* as an oil seed crop, the additional costs of solvent and phorbol ester recovery, however, pose hurdles that must be addressed if commercialization is to be realized.

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FIGURE CAPTIONS

Figure 1. Treatment pathway of *Jatropha* biomass.

Figure 2. Simplified pathway for *Jatropha* processing for mass balance.

Figure 3. Detailed pathway for *Jatropha* processing for energy balance

Table 1. Percent of Starting Material in End Phases

	Bio-oil (11)	Co-solvent (12)	Washed Methanol (22)	Bio-oil (25)	Bio-oil (31)	Seed Cake (28)	Total
Protein (BM) (%) ¹ :	0	12	1.3	0	0.004	86.6	99.9
Lipid (BM) (%):	55	0	0	14.9	20.6	0	90.5
Ionic liquid (CS) (%):	0.04	59.4	9.4	0.04	0	31	99.88

¹The percent yield of protein in the various phases are relative to the total amount of protein in the *Jatropha* seed prior to pretreatment

Table 2. Assumptions used in energy balance							
Parameter	Unit	Methanol	Hexane	EMIMAc	Seed Cake	Oil	Co-Solvent
Heat capacity, C_p	Btu/lb.F	0.6	0.54	0.6	-	-	-
Boiling point, Bp	F	148.3	155.8	-	-	-	-
Enthalpy of vaporization, H_{vap}	Btu/lb	473.44	157	-	-	-	-
Reaction temp	F	147.8	-	147.8	-	-	147.8
Room temperature	F	77	77	77			77
Densities	lb/gal	6.6	5.47	8.344	-	7.68	-
caloric value	Btu/lb	-	-	-	7824.53	17024.8	-
% in Co-solvent	%	70	-	30	-	-	-
Solvent to Biomass ratio	lbs/lb	1.32	3.96	-	-	-	1.32
Extraction/Recovery Efficiencies	%	100	100	100	100	100	100

TABLE 3: Energy Calculations		Amount Processed per year (lbs)	Energy (Btu/yr)	Process Requirement (ton/day)	Equipment capacity (ton/day)	Hours of operation (yr)	Power consumption specifications		# of Units required
Process #	Description						(kW)	(kWh/ton)	
1	Jatropha seeds	1.25E+08							
1A	Power supply for Flaker		1.57E+09	250	300	5000	92.02		1
2	Flaked jatropha seeds	1.25E+08							
3	co-solvent transfer to extractor	1.65E+08							
4	Heat supply for Extractor		1.24E+09	580	200	5800		2.5	3
5	Co-solvent-delipified seed for Centrifuge	2.46E+08	8.08E+08	492.5	873	3385	70		1
6	Crude jatropha oil	4.38E+07	7.45E+11						
7	Wet Detoxified seed cake	1.01E+08							
8	Phorbol ester with co-solvent	1.33E+08							
9	Heat supply for Evaporator 1		8.96E+08	265.2	33.3	5968	5.5		8
10	Recovered methanol	1.01E+08	5.23E+10	Calculated value: condensed from bp to 25°C.					
11	Phorbol ester with EMIMAc	4.41E+07							
12	Recovered EMIMAc is recycled	4.35E+07							
13	Phorbol ester with organic solvent	3.10E+06							
14	Heat supply for Evaporator 2		2.09E+07	6.2	33.3	1116	5.5		1
15	Recovered solvent is recycled	2.48E+06	4.94E+08	Calculated from hexane values: condensed from bp to 25°C.					
16	Crude phorbol ester recovered	6.25E+05	4.89E+09						
17	Methanol from storage tank	2.18E+08							
18	MeOH rinse to mixing tank	1.22E+08							
19	Excess MeOH rinse to storage tank	1.84E+08							
20	Wet non-toxic seed cake for purification	1.01E+08							
21	Heat supply to DTDC		2.58E+09	202	10-3000			15	1
22	condensed MeOH for storage	2.02E+07	1.04E+10	Calculated value: condensed from bp to 25°C.					
23	Dried cake for grinding	8.96E+07							
24	Heat supply for Grinder		2.03E+09	179.2	39.6	5418	22		5
25	Ground seed cake for animal feed	8.96E+07	7.01E+11						
26	MeOH make up								
27	EMIMAc make up								
	Energy Balance for Extraction process		1.38E+12

Figure 1

Figure 3