# Plant-based Hydrolysates as Effective Replacements for Animal Hydrolysates in Microbial Fermentation Applications

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## Broader Context

- Hydrolysate, an enzymatic digest of bulk protein sources, is widely utilized as the major carbon and nitrogen source for a wide variety of bacterial media formulations (Difco Manual 2nd edition 2009). Plant and yeast-based hydrolysates provide a sustainable, cost-effective, lower risk, and ethical alternative to animal-derived hydrolysates and extracts.
- Between hydrolysate types, the variability of free amino acids and small oligopeptides, as well as trace metals and carbohydrate content, can contribute to the suitability of different hydrolysates for each microbes' varying metabolic pathways.
- As 70-80% of Nu-Tek BioSciences plant and yeast hydrolysate is composed of peptides 1-6 amino acids in length, percent free amino acid and total nitrogen content is one method of comparing nutrient availability between hydrolysates (Figure 1 and Table 1).

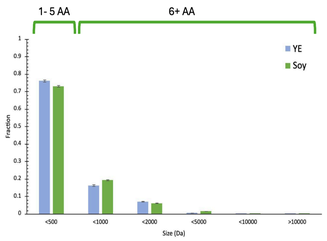


**Figure 1.** Size exclusion chromatography fractionation of hydrolysate using HPLC. Mobile phase used is 30% ACN:70% $H_2O$: 0.1% trifluoroacetic acid run on an SEC column from Tosoh Biosciences (Tokyo, Japan).

**Table 1.** Table of amino acid supply and demand for different hydrolysates. Shown is a representative total AA demand for *E. coli* to be met by free AA from different hydrolysates. Amounts are in g/100 g.

| AA | E. coli | Soy Flour | Pea Flour | Yeast Extract | Tryptone |
|---|---|---|---|---|---|
| G, Gly | 7.5 | 0.17 | 0.54 | 1.04 | 2.2 |
| L, Leu | 10.2 | 2.05 | 5.04 | 3.15 | 8.4 |
| K, Lys | 4.7 | 1.55 | 1.15 | 1.87 | 7.5 |
| V, Val | 7.3 | 0.94 | 2.51 | 2.36 | 5 |
| D, Asp* | 5.3 | 0.67 | 1.13 | 1.77 | 6.3 |
| E, Glu** | 6.1 | 1.77 | 3.03 | 5.65 | 21.1 |
| T, Thr | 5.3 | 0.99 | 3.13 | 2.25 | 4.2 |
| F, Phe | 3.8 | 1.53 | 3.75 | 2.09 | 4.6 |
| N, Asn* | 3.8 | * | * | * | * |
| I, Ile | 5.9 | 0.85 | 2.43 | 1.87 | 5.6 |
| Y, Tyr | 1.3 | 0.17 | 0.09 | 0.47 | 6.1 |
| S, Ser | 5.5 | 0.59 | 1.77 | 1.75 | 5.6 |
| H, His | 2.3 | 0.41 | 0.99 | 0.5 | 2.7 |
| P, Pro | 4.4 | 0.35 | 0.4 | 0.95 | 9.9 |
| A, Ala | 9.7 | 0.68 | 1.46 | 3.67 | 2.8 |
| Q, Gln** | 4.3 | ** | ** | ** | ** |
| R, Arg | 5.8 | 0.44 | 3.34 | 1.56 | 3.6 |
| M, Met | 2.8 | 0.41 | 0.28 | 0.71 | 2.7 |
| C, Cys | 1.2 | 0.08 | 0.14 | 0.11 | 0.3 |
| W, Trp | 1.3 | 0.28 | 0.41 | 0.57 | 1.1 |

**Table 2.** ACDF hydrolysates utilized in these studies. All are products from Nu-Tek BioSciences.

| Hydrolysate Source | Product |
|---|---|
| Soy Flour | Soy Peptone HSP-A |
| Soy Isolate | Soy Peptone HSP-I |
| Soy N | Soy Peptone HSP-N |
| Pea Isolate | Pea Peptone HPP-A |
| Yeast Extract | Yeast Extract FP100 |
| Wheat | Wheat Hydrolysate HWP-A |

- Tryptone, tryptose, porcine hydrolysate, and beef extract are animal-derived (AO) protein sources utilized as the major component within common media formulations such as Lysogeny broth (LB), tryptose phosphate broth (TBP), tryptic soy broth (TSB), soy peptone agar (SPA) and de Man, Regosa and Sharpe broth (MRS).
- Optimal ACDF hydrolysate replacement for AO protein sources was evaluated for a panel of industry-representative gram-positive and gram-negative bacteria, various lactic acid bacteria, and *E. coli* strains, using biomass growth methods and pDNA yield (*E. coli* ).

**Table 3.** Formulation of MRS broth used in *L.casei, L.acidophilus,* and *L.lactis* growth curves. All gram amounts are per liter.

| Component | AO | ADCF |
|---|---|---|
| Proteose Peptone No. 3 | 10.0 g | - |
| Beef Extract | 10.0 g | - |
| ADCF Hydrolysate | - | 20 g |
| Yeast Extract | 5.0 g | 5.0 g |
| Dextrose | 20.0 g | 20.0 g |
| Polysorbate 80 | 1.0 g | 1.0 g |
| Ammonium Citrate | 2.0 g | 2.0 g |
| Sodium Acetate | 5.0 g | 5.0 g |
| Magnesium Sulfate | 0.1 g | 0.1 g |
| Manganese Sulfate | 0.05 g | 0.05 g |
| Dipotassium Phosphate | 2.0 g | 2.0 g |

**Table 4.** Percent total nitrogen (% TN) of hydrolysates and the % w/v of each hydrolysate used in TN normalized MRS and LB. The % TN data was taken from certificates of analysis provided by manufacturers.

| Hydrolysate | %TN w/w | %w/v MRS | %w/v LB |
|---|---|---|---|
| Beef/Porcine | 12% | 2% | - |
| Tryptone | 12.4% | - | 1% |
| Soy Flour | 7.60% | 3.16% | 1.63% |
| Pea Isolate | 13% | 1.85% | 0.95% |
| Yeast Extract | 11% | 2.18% | - |
| Soy Isolate | 13.3% | - | 0.43% |
| Soy N | 9.10% | - | 1.36% |
| Soy:Pea Isolate | 13.15% | - | 0.47% |



## LAB Growth Curves

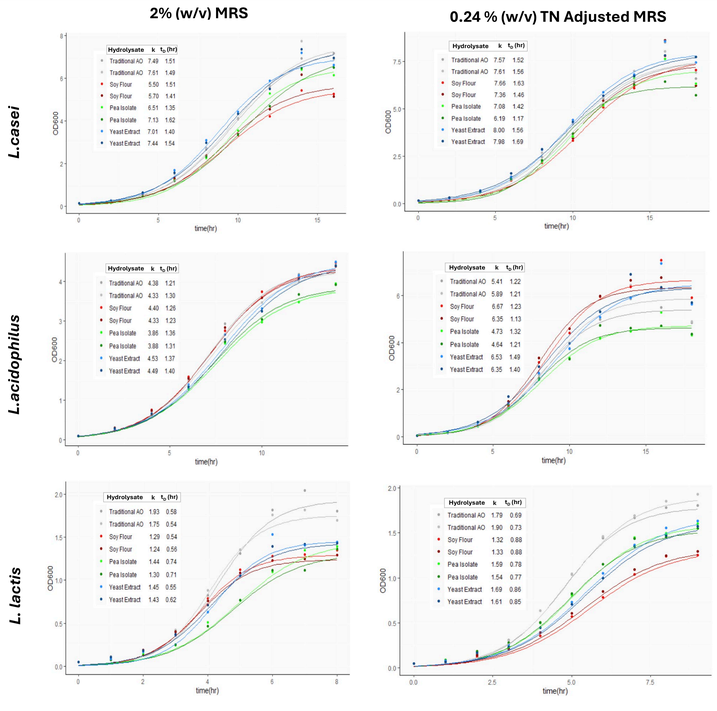


**Figure 2.** Growth curves of different lactic acid bacteria cultured in MRS broth based on a traditional or ADCF formulation. MRS formulations were prepared with either a 2 % (w/v) replacement of animal extracts for ADCF hydrolysate as given in Table 3; or with an MRS formulation normalized for the total % N from hydrolysate as in Table 4. In the case of *L. casei* and *L. acidophilus*, normalizing for % TN resulted in comparable or greater biomass using an ADCF hydrolysate versus beef and porcine extracts. The notably more fastidious *L. lactis*, when propagated in MRS, showed the lowest increase in biomass over all formulations with pea and yeast hydrolysates showing improvement with normalized % TN, potentially due to a larger supply of branched amino acids within the pea and yeast compared to other hydrolysates (Aller et al 2014).

## Experimental Methods

**Standard Growth Study:**

- *Lactobacillus acidophilus* (ATCC 314), *Lactococcus lactis (*ATCC 11454), *or Lactobacillus casei* (ATCC 393) was grown in 1X MRS formulation with the addition of hydrolysate as described in tables 3 and 4.
- An initial 10mL pre-growth liquid culture was inoculated and incubated at 37°C in liquid media for 8 hours to provide sufficient density for inoculation of a second 10mL pre-growth at 0.05 OD600. Cultures were inoculated after 12hrs in 10mLs of growth media.
- Culture growth was measured using a spectrophotometer at a 600nm wavelength every 1-2hrs to generate a standard growth curve using *Growthcurver* in R-studio (Sprouffske & Wagner, 2016).

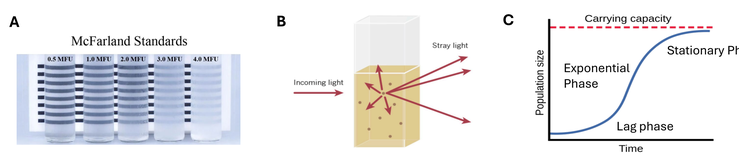


**Figure 3.** (A) MacFarland Standards used to measure turbidity in TSB/TPB liquid media. (B) Cuvette with bacterial culture used to measure increases in cellular density via light scattering. (C) Logistic pattern of bacterial growth curves measured using OD600 values in a UV-Visible Spectrophotometer.

**Microbial Biomass Study:**

- Inoculated 10mL TSB/TPB liquid media or SPA/SBA agar plates between 10-100cfu (*S. epidermidis* ATCC 14990 , *S. pyogenes* ATCC 19615 , *S. pneumonia* ATCC 6305 , *B. subtilis* ATCC 6051 , *E. coli* ATCC 8793), all microorganisms purchased as lyophilized pellets (Microbiologics, Saint Cloud MN).
- Incubated liquid media for 4 days, measuring density with McFarland turbidity standards at the 24hr mark.
- Incubated agar plates until visible colonies (up to 4 days) and marked colony number and average size as compared to TSA sheep blood agar control plate (Hardy Diagnostics, Santa Maria CA).

**Epi300 Growth and Plasmid Yield:**

- Epi300 *E. coli* with F-purple plasmid (Figure 1) were grown in various LB formulations, with an 8hr pregrowth followed by inoculation at OD600 0.05 for 12-14hrs (with addition of inducer, copy control). Each 5mL culture is pelleted, washed with 1X PBS, and frozen at - 80°C.
- DNA standards isolated with QIAGEN Dneasy Blood and Tissue kit and QIAprep Spin Miniprep kit (QIAGEN, Venlo Netherlands) and quantified using Nanodrop 2000 (Thermo Fisher, Waltham MA).
- In-lysis qPCR (methodology from Song et al 2021) with addition of 10^4 cells/mL and 0.1% IGEPAL to each rxn, targeting chloramphenicol resistance gene (cmr) on pCC1Fos plasmid, and dnaB gene on chromosomal DNA.

## *E. coli* Biomass and pDNA

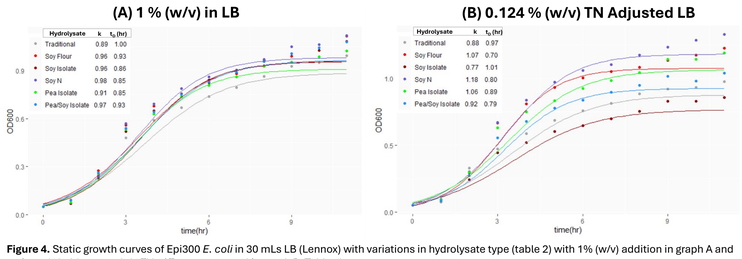


**Figure 4.** Static growth curves of Epi300 *E. coli* in 30 mLs LB (Lennox) with variations in hydrolysate type (table 2) with 1% (w/v) addition in graph A and various %(w/v) to match % TN of Tryptone control in graph B (Table 4).

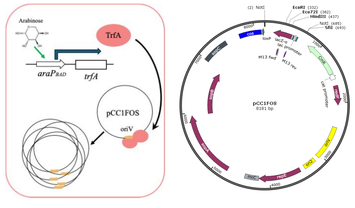


**Figure 5.** A diagram of the components of the pCC1FOS F-purple. Contains 2 origins of replication allowing for copy number control by L-arabinose induced system. pCC1FOS includes chloramphenicol resistance gene and violacein operon.

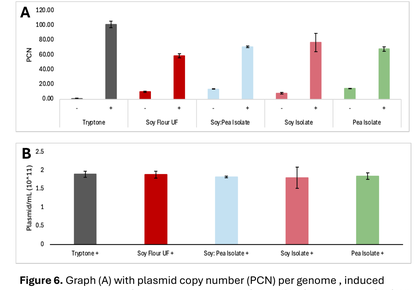


**Figure 6.** Graph (A) with plasmid copy number (PCN) per genome , induced cultures as (+) and noninduced cultures as (-). Graph (B) with total plasmid yield with induction for different peptones at 1% w/v with 250rpm shaking at 37 °C.

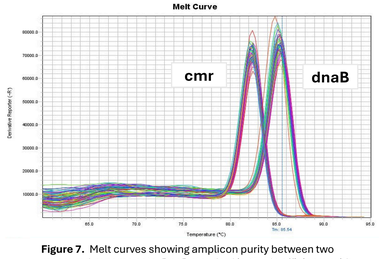


**Figure 7.** Melt curves showing amplicon purity between two measured genome sets. DnaB standard is 95.5% efficiency with $R^2$:1. Cmr standard is 92.5% efficiency with $R^2$:1.

| A Hydrolysate | Cells10^9/mL |
|---|---|
| Tryptone - | 2.75 |
| Tryptone + | 1.88 |
| Soy Flour UF - | 4.19 |
| Soy Flour UF + | 3.21 |
| Soy: Pea Isolate - | 3.26 |
| Soy: Pea Isolate + | 2.58 |
| Soy Isolate - | 3.41 |
| Soy Isolate + | 2.35 |
| Pea Isolate - | 3.22 |
| Pea Isolate + | 2.72 |

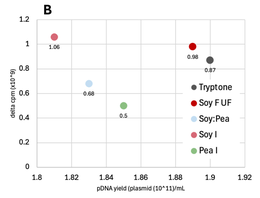


**Figure 8.** Table A denotes Epi300 cell density at time of harvest. Figure B denotes metabolic burden response to induction of pDNA (copy number increase). Y-axis notes cells per mL (cpm) as a difference in cell density values with and without inducer as a measure of metabolic burden. X-axis plotted as a function of plasmid yield.

## Microbial Biomass

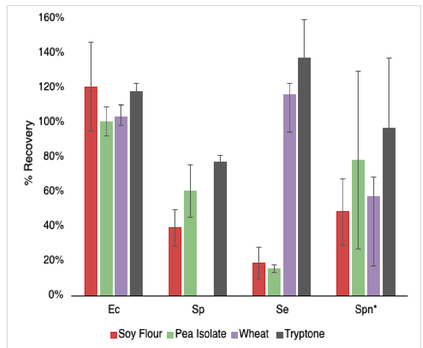


**Figure 9.** Percent recovery of colonies compared to control agar plate. Microbes tested include *S. epidermidis* (Se), *S. pyogenes* (Sp), *S. pneumonia* (Spn), and *E. coli* (Ec).

**Table 5.** All gram amounts are per liter. (A) Formulation of Soy Peptone Agar (SPA/SBA) plates used to test growth of microbes. Sheeps' Blood was added to the plates used with Spn. (B) Formulation of Tryptic Soy Broth (TSB) tested as a liquid culture. (C) Formulation of Tryptic Phosphate Broth (TPB) tested as a liquid culture.

| A Component | AO | ADCF |
|---|---|---|
| Tryptone | 20.0 g | - |
| ADCF Hydrolysate | - | 20 g |
| Sodium Chloride | 3.0 g | 3.0 g |
| Agar | 10.0 g | 10.0 g |
| *Sheeps' Blood (used with Spn) | 50.0 ml | 50.0 ml |

| B Component | AO | ADCF |
|---|---|---|
| Tryptone | 17.0 g | - |
| Soy Hydrolysate | 3.0 g | - |
| ADCF Hydrolysate | - | 20 g |
| Dextrose | 2.5 g | 2.5 g |
| Sodium Chloride | 5.0 g | 5.0 g |
| Dipotassium Phosphate | 2.5 g | 2.5 g |

| C Component | AO | ADCF |
|---|---|---|
| Tryptone | 20.0 g | - |
| ADCF Hydrolysate | - | 20 g |
| Dextrose | 2.0 g | 2.0 g |
| Sodium Chloride | 5.0 g | 5.0 g |
| Disodium Phosphate | 2.5 g | 2.5 g |

| A TSB | Soy Flour | Pea Isolate | Wheat | Yeast Extract | Tryptone |
|---|---|---|---|---|---|
| Ec | ✓ | ✓ | ✓ | ✓ | ✓ |
| Bs | ✓ | ✓ | ✓ | ✓ | ✓ |
| Se | x | x | ✓ | ✓ | ✓ |
| Sp | ✓ | ✓ | ✓ | ✓ | ✓ |

| B TPB | Soy Flour | Pea Isolate | Wheat | Yeast Extract | Tryptone |
|---|---|---|---|---|---|
| Ec | ✓ | ✓ | ✓ | ✓ | ✓ |
| Bs | ✓ | ✓ | ✓ | ✓ | ✓ |
| Se | ✓ | x | ✓ | ✓ | ✓ |
| Sp | ✓ | ✓ | ✓ | ✓ | ✓ |

**Figure 10.** Turbidity of microbes in (A) TSB and (B) TPB media were measured using McFarland Standard. Hydrolysates that had a 4+ turbidity within 24 hours passed the test. Microbes tested include *S. epidermidis* (Se), *S. pyogenes* (Sp), *B. subtilis* (Bs), and *E. coli* (Ec). Pea Isolate TSB and TPB

## Conclusions/Future Inquiries

**Conclusions:**

- In microbial biomass growth tests, plant-derived hydrolysates can successfully replace animal derived components in a panel of representative gram-negative and gram-positive bacteria.
- The efficacy of plant-derived hydrolysates was confirmed in lactic acid bacteria growth curves, showcasing the effects of total nitrogen adjustment to compare differences in the hydrolysate types beyond peptide availability.
- Soy flour LB is equivalent to tryptone LB in total plasmid yield from Epi300 *E. coli,* with soy flour LB supporting a higher density of cells to even out the higher specific yield from tryptone LB.

**Future Inquiries:**

- While total nitrogen is an estimate of protein availability for cellular metabolism, testing carbon/nitrogen (C/N) ratios can showcase differences in carbohydrate content as well as protein availability between hydrolysate media formulations.
- While MRS is the optimal media formulation for Lactobacilli such as *L. casei* and *L. acidophilus*, M17 is the medium of choice for *Lactococcus* genus such as *L. lactis* (He et al 2025). Testing hydrolysate supplementation in M17 along with hydrolysate blending studies would be the next steps to develop a sustainable media that fulfills the metabolic needs of *L. lactis*.
- While direct supplementation allowed equal performance between tryptone and soy flour in plasmid production, this same experiment will be repeated with total nitrogen (TN) adjusted media to further optimize plasmid production in ACDF hydrolysate media.

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