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RESEARCH PAPER

In vitro Digestibility of *Yarrowia lipolytica* Yeast and Growth Performance in Whiteleg Shrimp *Litopenaeus vannamei*

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Abstract

Marine yeasts used in aquaculture disease control can also be an important protein source for improving feeding and nutrition of crustaceans. *Yarrowia lipolyticca* has been studied for its capacity to secrete heterologous proteins and high content of unsaturated fatty acids, beta-glucan, and mannane polymers in the cell wall. We measured *in vitro* digestibility of *Y. lipolyticca* by whiteleg shrimp *Litopenaeus vannamei* digestive enzymes, and an *in vivo* assay of *Y. lipolytica* in feed on whiteleg shrimp growth. We found that digestive gland enzymes of shrimp digest *Y. lipolytica*, based on reduced optical density of a yeast suspension. Digestion was –0.00236 ± 0.00010 OD U min^{-1} for intact cells and –0.00325 ± 0.00010 OD U min^{-1} for lysed cells. Release of reducing sugars in intact cells (5.3940 ± 0.1713 µmol h^{-1}), and lysed cells (0.8396 ± 0.2251 µmol h^{-1}) was measured. Digestive gland treatment significantly reduced cell viability (near 100%), relative to the control. Electron microscopy shows that the cell wall of *Y. lipolytica* exposed to the digestive gland enzymes was severely damaged. Shrimp diet containing *Y. lipolytica* resulted in significantly higher weight gain and specific growth rate of whiteleg shrimp.

Keywords: Marine yeast; cell digestibility; cell viability; turbidimetry; reduced sugars.

Introduction

Fish meal and oil to supply feed for shrimp aquaculture has declined in recent years because extraction of fish is reaching exploitation limits (Valenzuela, 2012). This has led the industry to seek alternative feed ingredients, including vegetable sources (Douglas, 2010), which is limited by the presence of anti-nutritional factors, such as a trypsin inhibitor (Urán *et al*., 2009). Another cost factor is the spread of diseases primarily caused by viruses and bacteria, which affect the organisms under cultivation, caused serious economic losses, company closures, and unemployment (Aguirre *et al*., 2000; Bustillo-Ruiz *et al*., 2009; Godínez *et al*., 2012). An alternative strategy for disease control and better nutritional quality of shrimp and fish feed is the use of yeast or yeast fractions (Guzmán *et al*., 2007; Tovar *et al*., 2008). Yeast have excellent nutritional content and functional properties, including a role as probiotics and immune-stimulants (Navarrete *et al*., 2012; Tovar *et al*., 2002). It is available as a powder or liquid suspension. Including brewer yeast in feed increased growth and disease resistance in tilapia (Abdel *et al*., 2008) and hybrid striped bass (Li & Gatlin, 2004). For shrimp, yeast or their fractions in feed improved resistance to vibriosis (Burgents *et al*., 2004; Scholz *et al*., 1999) and white spot syndrome virus (WSSV) (Babu *et al*., 2013). A yeast-based protein replaced fish meal in a whiteleg shrimp diet without affecting growth (McLean *et al*., 2006).

Yarrowia lipolytica is a yeast-like species that metabolizes hydrocarbons and lipids and secrete heterologous proteins (Barth & Gaillardin, 1996; Dominguez *et al*., 2000). It is rich in lysine, phenylalanine, valine, tryptophan, isoleucine, and vitamins (B1, B2, biotin, folic acid, nicotinic acid, choline, niacin) and its protein content reaches 80% of shrimp requirements (Ruiz *et al*., 2003). It also contains unsaturated fatty acids, mainly *linoleic* acid, and α-linolenic acid (Athenstaedt *et al*., 2006; Beopoulos *et al*., 2008; Ratledge**,** 2005), essential for penaeid shrimp (Shiau, 1998)**.** Unsaturated fatty acids are more easily digested, compared to saturated fatty acids, based on forming micelles that are easily absorbable (British Nutrition Foundation, 2013). The yeast cell wall contains 70% neutral carbohydrate, 7% amino sugars, 15% protein, 5% lipids, and 0.8% phosphorus; mycelial cell walls contained 70% carbohydrate, 14% amino-sugars, 6% protein, 5% lipids and, 6% phosphorus (Vega & Domínguez, 1986). Three main polysaccharides present in the *Y. lipolytica* cell wall are β-glucans, mannans, and chitin, which play a role in modulating the crustacean

immune response (Chang *et al*., 2003; Dobrescu, 2002). *Y. lipolytica* also acts as a prebiotic because the glucans and mannans stimulate the shrimp immunological system through the reticuloendothelial system (Vega & Domínguez, 1986; Vetvicka *et al*., 2013). In general, yeast β-glucan is a modulator of immunological response at the cellular and humoral (antibody-mediated) level (Raa, 2015).

The nutritional benefits that *Y. lipolytica* can bring in the nutrition of shrimp is largely unknown because we do not know if the digestive enzymes in shrimp are able to degrade the cell wall of *Y. lipolytica* and act as a probiotic food source in aquaculture. This study determined whether the digestive enzymes of *L. vannamei* degrade the cell wall of *Y. lipolytica* and how its use as a supplement or basic feed affects shrimp growth. To our knowledge, this is a novel use, allowing us to determine whether *Y. lipolytica* should be considered an additive in balanced shrimp diets.

Materials and Methods

Enzymatic Extracts from Shrimp Digestive Tract

We randomly obtained 66 juvenile whiteleg shrimp (*Litopenaeus vannamei*) from a local farm pond at intermolt stage with average weight of 5.6 ± 1.2 g and length of 6.3 ± 0.64 cm. The shrimp were apparently healthy, with hard, turgid bodies. Their appendices were intact, and without stains or injuries to the exoskeleton. Dissections were made in their digestive tract, separating stomach, digestive gland, and anterior, middle, and posterior intestine. The tissues of each segment were weighed and separately homogenized with cold distilled water (0 °C) in a v/w proportion of three mL water g^{-1} fresh tissue. Raw extracts were centrifuged at 15,294 *g* for 10 min at 4 °C. The lipid fraction was removed and the supernatant was recovered and stored at –20 °C, for later analysis of protein and digestive enzyme activity. Enzymes were also used in the digestibility study.

Yeast Cultivation and Cell Recovery

The marine yeast *Yarrowia lipolytica* (Y1BCS033), isolated and characterized at CIBNOR, was used in the digestibility study after 16 h growth in YPD liquid medium at 28 °C and stirred at 200 rpm (Barth & Gaillardin, 1996). Cells were recovered by centrifugation at 15, 300 *g* for 10 min at 4 °C (5810R, Eppendorf, Hamburg, Germany). The cells were washed three times with sterile 0.9% saline water and then resuspended in sterile saline water. The cell suspension optical density (630 nm) was adjusted to 1.75 units, and kept at 4 °C. The cell suspension was used for further experiments on cell digestibility and viability.

Yeast Cell Disruption

Washed yeast cells (3 mL) were lysed (Hoffman & Winston 1987) by a cell disrupter containing 3 g glass beads of three sizes (0.1 mm, 1.0 mm, and 3.5 mm; 1 g each), and 200 µL of lysis buffer (1% SDS, 100 mM NaCl, 10 mM Tris-HCl at pH 8.0 and 1 mM EDTA) for six periods of 30 s, interspersed by an ice water bath lasting 30 s. The cell fractions were recovered by centrifugation for 1 h at 15, 300 *g* at 4 °C. The pellets were washed three times with 500 mL 0.9% sterile saline water. Lysed cells were resuspended in sterile saline water at optical density of 1.75 units at 630 nm, kept at 4 °C, and the lysate stored at –20 °C for futures experiments.

Yarrowia lipolytica Cell Digestibility by Shrimp Enzymes (Optical Density Method)

In a 96-well plate, the reaction mixture, composed of 50 µL Tris-HCl buffer (100 mM at pH 8.0), 100 µL intact cell suspension, and 25 µL shrimp enzymatic reagent (stomach, digestive gland, anterior intestine, middle intestine, or posterior intestine). The decrease in optical density at 630 nm was recorded every minute for the first 20 min, and then every 5 minutes to complete a one-hour digestibility assay. Additionally, four control treatments were performed: (1) Reaction mixture without shrimp enzyme reagent, (2) Reaction mixture without cell suspension, and (3) Reaction mixture with inactivated shrimp enzyme reagent. The same experiment was done replacing intact cells by lysed cells. In all treatments, the final volume of reaction mixture was 175 uL and optical density at 630 nm close to 1.0 unit at time zero. The results were adjusted by subtracting the decrease of optical density (OD) in control 1 (without the enzymatic agent), and expressed as units of decreased OD min^{-1}.

The shrimp degrading activity on yeast cells (DAYC) was calculated for each digestive tract section. One unit of DAYC was considered as the enzyme required to decrease OD in 0.0001 Abs units at 630 nm min^{-1}. Total activity was calculated and expressed as percent of activity within each digestive tract section.

Yarrowia lipolytica Cell Digestibility by Shrimp Enzymes (Released Reduced Sugars Method)

Released reduced sugars from shrimp enzymatic hydrolysis of *Y. lipolytica* cells was measured (Vega *et al*., 1993) and modified as follows: In test tubes, 250 µL Tris-HCl buffer (pH 8.0, 50 mM), 100 µL reaction mixture substrate (containing intact or lysed cells), 25 µL shrimp enzyme reagent (stomach, digestive gland, anterior intestine, middle intestine, or posterior intestine) were added. The mixture was hand-shaken, and the tubes, laid at 30°, were incubated and stirred at 120 rpm at 25 °C (room

temperature) for 4 h. The reaction was stopped by adding 200 µL 2N sodium carbonate and 1.5 mL DNS reagent. The reaction mixture was vortex-shaken and incubated at 95.5 °C in a water bath for 15 min. Then, 7.3 mL distilled water was added and absorbance was spectrophotometrically measured at 550 nm (#6505 UV/VIS, Jenway, Bibby Scientific, Stone, Staffordshire, UK). The blanks were treated in the same manner, adding enzyme reagent after the reaction was stopped by 2N sodium carbonate and DNS reagents. Glucose (G5767, Sigma-Aldrich) was used as the standard. The results were adjusted, subtracting the released reducing sugars by control 1 (without the enzymatic agent) and expressed as µmol of released reducing sugar hour^{-1}.

The reducing shrimp sugar releasing activity on yeast cells (RSRYC) was calculated for each digestive tract section. One unit of RSRYC was considered as the enzyme required to release 0.1 µmol of reducing sugars from yeast cells hour^{-1}. Total activity was calculated and expressed as percent activity within each shrimp digestive tract section.

Effect of Shrimp Enzymatic Treatment on *Yarrowia lipolytica* Viability

The marine yeast *Y. lipolytica* cell suspension (YCS; 500 µL) was used as the substrate with 500 µL of shrimp enzymatic reagent (stomach, digestive gland, anterior intestine, middle intestine, or posterior intestine), The reagent had been sterilized by filtration (sterile Corning RC filters, 0.2 µm pore). The reaction mixture was incubated in test tubes resting at a 30° angle at room temperature for 4 h. After incubation, decimal dilutions were performed (1×10^{-1} to 1×10^{-5}), starting with 100 µL reaction mixture and 900 µL sterile 0.9% saline solution. From each dilution, 100 µL was spread on Petri dishes containing YPD solid medium, then incubated at 28 °C for 24 h. Counts (CFU) were made, considering colony morphology of the *Y. lipolytica* strain and selecting dilution with 25–250 CFU for counting and transforming the results to log UFC mL^{-1} (Swanson *et al*., 2001; Camacho *et al*., 2009). Each treatment was performed in triplicate, including control treatments under the same experimental conditions, but using 500 µL sterile saline solution, rather than an enzyme reagent.

Analysis of *Yarrowia Lipolytica* Cells with Electron Microscopy

YCS (500 µL) was placed in a test tube with 500 µL enzyme reagent from the stomach, digestive gland, anterior intestine, middle intestine, or posterior intestine. The reaction mixture was incubated in tubes resting at a 30° angle for 4 h at room temperature and rotated at 120 rpm. Samples (100 µL) were taken at 0, 0.5,1, 2, 3, and 4 h; 50 µL fixative solution (2.5% glutaraldehyde) was added and let stand for 40 min at room temperature. *Y. lipolytica* cells were recovered by centrifugation at 15,300 *g* for 10 min at 4 °C, and washed three times with 50 mL 0.9% saline solution. As described by Linares (2007), the washed cells were treated with 100 µL osmium tetroxide (OsO_4) buffer (201030**,** Sigma-Aldrich) for 48 h at room temperature. Then, three washes with 50 mL 0.9% saline solution by centrifugation (15,300 *g* for 10 min at 4 °C). The cell pellets were subsequently immersed in 100 µL alcohol at gradients of 20–100% (v/v) at intervals of 30 min. On a sample holder on a microscope slide with double-sided tape, 10 µL of the alcohol-dehydrated yeast sample was placed and dried for 48 h on a heating plate. A gold coating was applied for 35 sec at 40 mA (Desk.11, Denton Vacuum, Moorestown, NJ) and the yeast cells were observed under an electron microscope (S-3000N, Hitachi High Technologies, Tokyo, Japan). Control treatments where prepared under the same experimental conditions, but with 0.9% saline solution instead of an enzyme reagent.

Feeding Trial with *Yarrowia lipolytica* Cells

One commercial diet was formulated (Akiyama *et al*., 1991). The diet contained 36% crude protein and 8% lipids (Table 1). Ingredients were pulverized and sieved through a 250 µm mesh. The dry ingredients were mixed in a blender to obtain a homogeneous mixture before the oil-based ingredients were added. Water was added (~30% of the mix weight) and mixed to obtain a firm paste. The mixture was passed through 2 mm die in a meat grinder. The pellets were dried in a forced-air oven at 45 °C for 12 h. A trial diet was prepared by adding 2% (v/w) of the *Y. lipolytica* solution at a concentration of 8000 cells mL^{-1} to reach a final concentration of ~0.8 g *Y. lipolytica* cells 100 g^{-1} feed). Diet containing *Y. lipolytica* was prepared once a week and stored at 4 °C until feeding time. Proximate analysis of the reference diet was conducted according to AOAC (2005) methods, and gross energy was measured with an adiabatic calorimeter. The feeding trial was conducted for 30 days. The reference and *Y. lipolytica* diets were tested in triplicate in tanks containing 40 L filtered seawater and 10 juvenile shrimp (initial weight 0.95 ± 0.11 g per tank. The feed ration for both treatments was served *ad libitum*, with a daily adjustment based on consumption. Water exchange (70%) was performed daily. Water temperature (28 ± 0.9 °C) was monitored daily. pH (7.9 ± 0.1), NH_3, NH_4^+ (<0.5 mg L^{-1}), NO_2 (<0.25 mg L^{-1}), and NO_3 (mg L^{-1}) was monitored weekly. At day 30 of the feeding trials, feed consumption (FC), final weight (FW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival were recorded.

Statistical Analysis

We used one-way ANOVA to analyze the data.

Table 1. Reference diet formula and proximate composition

Ingredient	Reference diet (g kg^{-1})
Fish meal [a]	240
Soybean meal [b]	240
Wheat meal [c]	391
Corn gluten [d]	20
Fish oil [e]	24
Soy lecithin [f]	40.8
Vitamin premix [g]	18
Trace mineral premix [h]	5
Vitamin C [i]	1
Alginic acid [j]	20
Antioxidant BHT [k]	0.2
Proximate analysis (dry basis)	
Moisture (%)	9.8±0.08
Protein (%)	36.0±0.04
Lipids (%)	8.2±0.18
Crude fiber (%)	1.1±0.06
Ash (%)	7.1±0.03
NFE (%)	46.7
Gross energy (cal/g)	4557±4.5

[a] Menhaden meal, Omega Protein Company, Luisiana, US.
[b] Promotora industrial acuasistemas SA de CV (PIASA), Baja California Sur, MX.
[c] Molino San Cristobal, Sonora, MX.
[d] Agro Insumos Basicos, SA de CV, MX.
[e] Proteinas marinas y agropecuarias, SA de CV· Jalisco, MX.
[f] Suministros AZ, BCS, MX.
[g] Vitamins: Vit. A, (20,000 UI/g) 90 mg/kg; Vit. B1, 9 mg/kg; Vit. B2, 54 mg/kg; Vit. B5, 90 mg/kg; Vit. B6, 18 mg/kg; Vit. B12, 0.04 mg/kg; Vit. K3, 36 mg/kg; Vit. D3, (850,000 UI/g) 144 mg/kg; Vit. H, 1 mg/kg; folic acid, 3.24 mg/kg; Inositol, 90mg/kg. Sigma aldrich, Missouri, US.
[h] Minerals: $CoCl_2$, 20 mg/kg; H_2MnO_5S, 3.3 g/kg; $H_{14}O_{11}SZn$, 66 g/kg; $CuH_{10}O_9S$, 1.3 g/kg; $FeSO_4$, 20 g/kg; Na_2SeO_3, 50 mg/kg; KI, 330 mg/kg. Sigma aldrich, Missouri, US.
[i] Rovimix Stay C 35%, DSM, Heerlen, NL.
[j] Sigma aldrich, Missouri, US.
[k] ICN Biomedical Inc, Ohio, US.

Significant differences were set at $P<0.05$. Data were then analyzed by the Tukey multiple comparison test (95% confidence). Analysis was performed with Statistica 7.0 (Dell Statisca, Tulsa, OK).

Results

Optical Density Test of Digestibility of *Y. lipolytica*

Optical density decreased in *Y. lipolytica* cell suspensions during exposure to shrimp digestive enzymes, with greater decline in lysed cells (Figure. 1B) than intact cells (Figure. 1A); the decreasing rate of optical density units (OD U) was –0.00236 ± 0.00010 OD U min^{-1} for intact cells and –0.00325 ± 0.00010 OD U min^{-1} for lysed cells. The capability of digestive organs to reduce optical density from intact and lysed cells was: digestive gland > stomach > anterior intestine > middle intestine > posterior intestine. The DAYC was 685.0 U on intact cells and 933.9 U on lysed cells. The DAYC of stomach and digestive gland represented 96% the total (Figure 2).

Reducing Sugar Test of Digestibility of *Y. lipolytica*

There was an increase in release of reducing sugars from the *Y. lipolytica* cell suspensions during exposure to digestive shrimp enzymes, more so in lysed cells (Figure. 3B) than in intact cells (Figure. 3A). The rate of release was 0.8396 ± 0.2257 μmol h^{-1} for intact cells and 5.3941 ± 0.1713 μmol h^{-1} for lysed cells. The capability of digestive organs to release reducing sugar from intact and lysed cells was: digestive gland > stomach > anterior intestine > middle intestine > posterior intestine. The RSRYC on yeast cell was 238.0 U for intact cells and 1546.1 U for lysed cells. The RSRYC of stomach and digestive gland, represent 96% the total U (Figure 4).

Cell Viability Test of Digestibility of *Y. lipolytica*

Cell viability of *Y. lipolytica* was 7.3 log UFC mL^{-1} in the control treatments (yeast exposed to distilled water), 6.2 log UFC mL^{-1} (stomach), 1.9 log UFC mL^{-1} (digestive gland), and 6.7, 7.2, and 7.2 log UFC mL^{-1} (anterior, middle, and posterior intestine, respectively). Cell viability was severely affected by exposure to shrimp digestive enzymes, especially by the digestive gland treatment (only 0.38 ± 0.02% survival), compared with the controls. Ranking of capability to reduce cell viability was: digestive gland > stomach > anterior intestine > middle intestine > posterior intestine (Figure. 5).

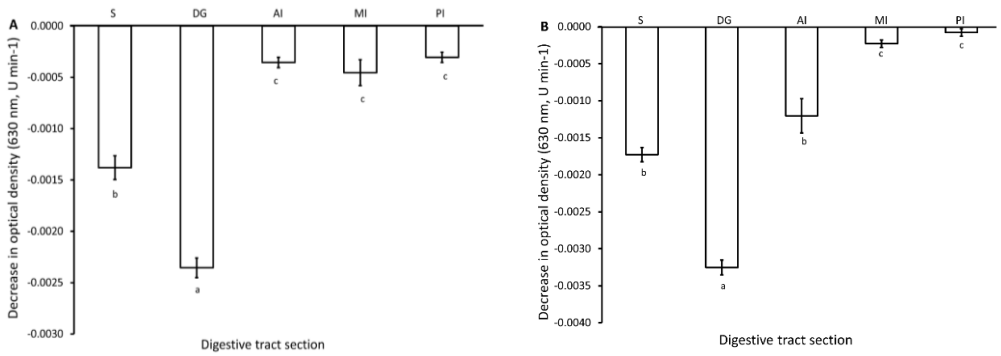

Figure 1. *In vitro* digestibility of *Y. lipolytica* by shrimp enzymes. The turbidimetric assay. A) Intact cell digestibility B) Lysed cell digestibility. Where: S) = stomach, (DG) = digestive gland, (AI) anterior intestine (MI) middle intestine, and (PI) posterior intestine.

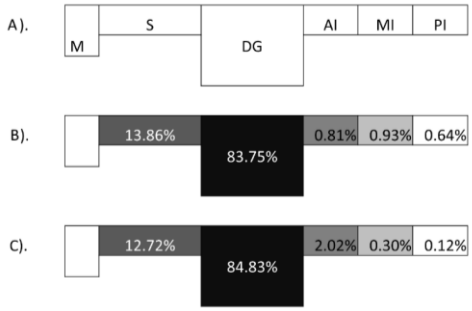

Figure 2. Distribution of digestive activity (%) from *Y. lipolitica* yeast in digestive tract of *L. vanammei*. A) M. Mouth, S, Stomach, DG, Digestive gland, AI, Anterior intestine, MI, Middle intestine, PI, Posterior intestine B). Intact cells were the 100% = 21.73 C) Lyzed cells were the 100% = 141.97.

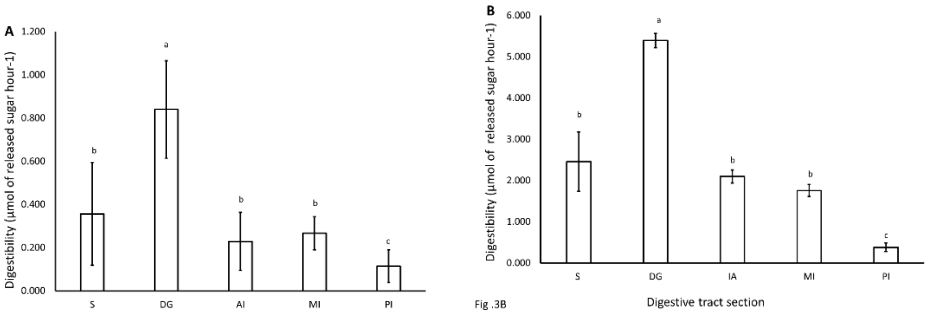

Figure 3. *In vitro* digestibility of *Y. lipolytica* by shrimp enzymes. Reducing sugar assay. A) Intact cell digestibility B) Lysed cell digestibility. Where: S) = stomach, (DG) = digestive gland, (AI) anterior intestine (MI) middle intestine, and (PI) posterior intestine.

Electron Microscopy Analysis

Electron microscopy analysis is shown in Figure. 6. For all treatments at time zero, *Y. lipolytica* cells had well defined structure and shape, without apparent cell wall damage. In the control treatment, at final time, cells were intact, with smooth cell walls and without apparent damage. Cells treated with stomach enzyme reagent at the end of treatment were damaged, resulting with cell fragments and extracellular material. At the end of digestive gland enzyme treatment, we observed many empty cells, severe damage, fissured cell, cell fragments, extracellular material, and some thin, irregularly shaped cells. Cells treated with anterior intestine enzyme reagent at the end of the treatment showed copious cell wall debris. Cells treated with enzyme reagent from the middle and posterior intestine had no irregularities at the end of the treatment.

Shrimp Feeding Trial

Shrimp performance after 30 days of feeding is presented in Table 2. Shrimp fed the diet with *Y. lipolytica* cells had significantly better weight gain and specific growth rate, compared to shrimp fed the

reference diet. Feed consumption, final weight, percentage of survival, and feed conversion ratio did not show significant differences among treatments.

Discussion

Based on optical density of cell suspensions, release of reducing sugars, cell damage (observed by electron scanning microscopy), and loss of cell viability, we determined that sections of the digestive tract of whiteleg shrimp digests cells of the marine yeast *Y. lipolytica*. The strongest digestion of yeast cells occurred with enzymes from the digestive gland, followed by the stomach, anterior, middle, and posterior intestine, respectively. The same pattern of digestibility occurred with all methods for intact or lysed cells, suggesting that *Y. lipolytica* nutrients are available for shrimp nutrition by digestion in stomach, digestive gland, and the anterior intestine. Higher digestibility of lysed cells agree with Zhao *et al*. (2015), finding that replacing fish meal by lysed yeast produced better results than intact cells**.** Forrellat *et al*. (1988) determined *in vitro* digestibility of eight protein sources by southern white shrimp *Litopenaeus schmitti*, finding that torula yeast, and a mixture of the Gram-positive bacteria *Celulomonas* sp. and brewer's yeast *Saccharomysescerevisiae* have digestibility of 32.66% and 43.90%, respectively. Chen and Co (1988) proposes 2% (w/w) yeast (no species mentioned) in shrimp feed for its positive effect on digestibility of nutrients, obtaining *in vitro* digestibility ranging from 83–87% for four species of penaeid shrimp *P. monodon* , *P. japonicus, P. semisulcatus* and *P. monoceros*, respectively.

Cell damage is the result of enzymatic digestion, with the strongest activity in the digestive gland (Omondi, 2005). Turbidity analysis is a very sensitive method for measuring biomass in cell cultures (Potvin *et al*., 1997). Kolmert *et al*. (2000) developed a quick and simple method for tracking sulfate-reducing bacteria in cultures, measuring optical density at 630 nm. In our study, turbidity analysis was modified to measure the decrease of optical density of intact or lysed *Y. lipolytica* cell suspensions by digestion of the cell wall by shrimp digestive tract enzymes. We found no variation in OD at 630 nm among the controls (1, 2, and 3) for turbidity test, resulting in a very low slope for intact cells (y = –0.0000200x, y = –0.0000634x, and y = –0.0000164x, respectively) and lysed cells (y = –0.000098x, y = –0.000052x, and y = –0.0000130). These results indicate that the decrease in optical density was caused by the hydrolysis capacity of shrimp digestive enzymes on the cell wall of *Y. lipolytica* and not by an external factor. These results are consistent with other studies that indicate that most of the digestion occurs in the digestive gland (Alexandre *et al*., 2014; Becerra *et al*., 2012; Hernandez & Murueta, 2009).

In aquaculture, yeast as a feed additive has been demonstrated in rotifers (Nagata & Whyte, 1992; Ryan, 2014), and growth of several penaeid shrimp species (Aguirre, 1994; Cornejo *et al*., 2015). One of the benefits of adding yeast to shrimp diets is its high content of β-glucan, which improves resistance against diseases (Chang *et al*., 2003; Sajeevan *et al*., 2006; Song *et al*., 1997; Sung *et al*., 1994;). For example, Sajeevan *et al*. (2009) obtained higher survival and immune response in the Indian white prawn *Fenneropenaeus indicus* challenged with WSSV after 40 days with a diet containing 10% whole cell biomass of the marine yeast *Candida sake S165* and a diet with 0.2% (w/w) β-glucan from the same yeast, compared to the control diet. Cornejo *et al*. (2015) used three experimental diets (3% activated yeast, 3% hydrolyzed yeast, and 0.6% β-glucan), resulting in an increase of hemocytes, significantly higher at 30 d from activated yeast (>3.3×10^6 cells mL^{-1}) than from hydrolyzed yeast (1.85×10^6 cells mL^{-1}) or ß-glucan (1.45×10^6 cells mL^{-1}). Sajeevan *et al*. (2009) challenged the Indian white prawn fed diets containing either 10% or 0.2% (w/w) yeast against WSSV, finding 96% mortality in prawns without yeast in the diet, 64% mortality in prawns fed 0.2% dietary yeast, and 36% mortality in prawns fed 10% dietary yeast. Deng *et al*. (2013) tested dietary yeast (0–0.15%, w/w) added to a commercial shrimp diet, with significant increases in final weight, survival, and FCR of *L. vannamei* fed yeast-enriched diets over the control diet. Yang *et al*. (2010) find a significant increase in weight gain, SGR, and survival of *L. vannamei* fed diets supplemented with red yeast *Rhodosporidium paludigenum* (1% dry yeast; 1×10^9 yeast cells g^{-1} diet), compared to the control. Gamboa *et al*. (2016) used torula yeast *Candida utilis* and fish meal in diets for *L. vannamei; t*he dietary nitrogen of the fish meal was replaced by increasing content of torula yeast (0 to 100%). Shrimp fed some torula yeast–fish meal diets had higher growth rates (k = 0.059–0.064) than shrimp fed diets containing only fish meal or only torula yeast (k = 0.041–0.054). Although, *Saccharomyces cerevisiae* yeast has been used in feed formulas for aquatic organisms (Aguirre *et al*., 2002), its application has been limited by the assumption of it having limited nutritional value and an indigestible cell wall (Lavens & Sorgeloos, 1996) or low palatability when using up to 5% in *L. vannamei* diets (Aguirre, 1994), 2% in *Macrobrachium amazonicum* diets (Hisano *et al*., 2008), or 0.5% in *M. rosenbergii* diets (Prasad *et al*., 2013). Compared with *Saccharomyces cerevisiae*, *Y. lipolytica* has higher content of lysine, phenylalanine, valine, tryptophan, isoleucine, B1, B2, biotin, folic acid, nicotinic acid, choline, and niacin (Ruiz *et al*., 2003). *Y. lipolytica* is also rich in the unsaturated linoleic and α-linolenic fatty acids (Ratledge**,** 2005; Athenstaedt *et al*., 2006; Beopoulos *et al*., 2008). Since unsaturated fatty acids are more easily digested than saturated fatty acids (British Nutrition Foundation, 2013), inclusion of *Y. lipolytica* should increase feed quality. Our results of shrimp enzymes

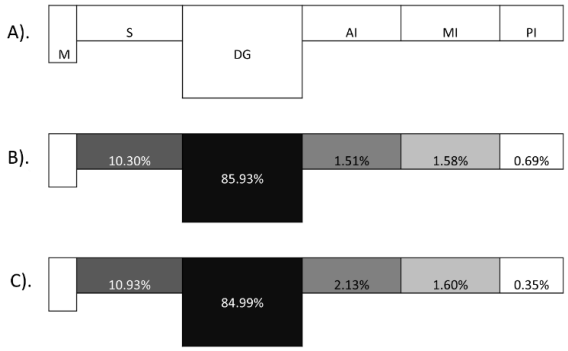

Figure 4. Distribution of digestive activity (%) for releasing reducing sugars from *Y. lipolitica* yeast in digestive tract of *L. vanammei*. A) M. Mouth, S, Stomach, DG, Digestive gland, AI, Anterior intestine, MI, Middle intestine, PI, Posterior intestine b). Intact cells were the 100% = 641.34 C) Lyzed cells were the 100% = 867.25.

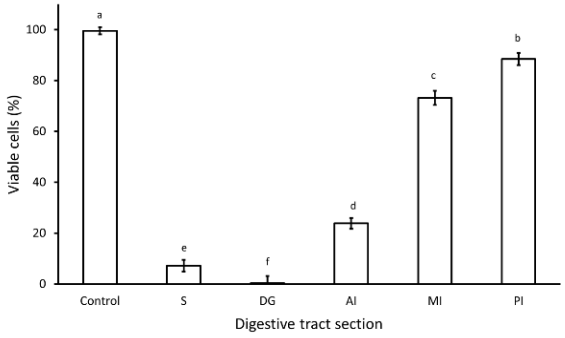

Figure 5. *Yarrowia lipolytica* cell viability after 4 h of exposure to ER from shrimp digestive tract. (S) = stomach, (DG) = digestive gland, (AI) anterior intestine (MI) middle intestine, and (PI) posterior intestine. 100% = 2.14 x10-8 ± 0.02 UFC/mL *Yarrowia lipolytica* cells.

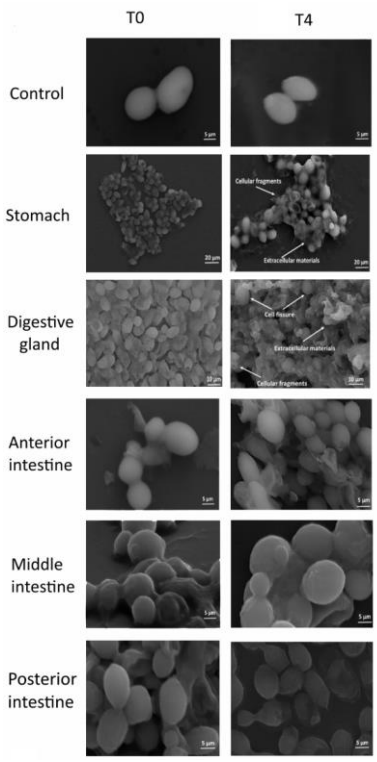

Figure 6. *Yarrowia lipolytica* cells at zero time (T0) and end time (T4 hours), after exposure to digestive enzyme of different sections of shrimp digestive tract (stomach, digestive gland, anterior intestine, middle intestine and posterior intestine. Pictures with 5 um, 10um or 20 um reference bars.

Table 2. Shrimp fed reference diet, and diet with *Y. lipolytica* during 30 days trial

	FC (g)	FW (g)	WG (%)	SGR (% day^{-1})	S (%)	FCR
Reference diet	3.82±0.29^{a}	3.80±0.03^{a}	297±4.2^{a}	4.59±0.04^{a}	93±2^{a}	1.36±0.03^{a}
Diet with *Y. lipolytica*	3.89±0.22^{a}	3.96±0.11^{a}	318±6.8^{b}	4.77±0.05^{b}	97±6^{a}	1.29±0.12^{a}

Values are given as mean ± SD of triplicate determinations. Means with different superscripts in same column are significantly different (P<0.05).
WG (%) = (final weight−initial weight)/ initial weight*100.
SGR (% day-1) =100 (ln average final weight−ln average initial weight) /number of days.
FCR = dry weight of pelleted feed consumed (g) /wet weight gain (g).
S (%) = final number of shrimp/ initial number shrimp *100.

on digestion time were similar to the results reported by Limsuwan (2009). Better growth with a feed supplement of *Y. lipolytica* should be expected for cultivated shrimp.

Conclusions

Yarrowia lipolytica marine yeast is efficiently digested by digestive enzymes of *L. vannamei*, including breakdown of its cell wall. Enzymes of the digestive gland had the strongest digestion power on intact and lysed yeast cells. Shrimp receiving this yeast in their diet had higher growth. *Y. lipolytica* yeast should be considered a useful additive in commercial shrimp diets.

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