Efficacy of co-fermented vegetable discard as shrimp feed ingredient

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ABSTRACT

A study was carried out wherein mixed vegetable discards from the local market were fermented using the bacterium *Bacillus licheniformis* MTCC 6824, for 5 days. *B. licheniformis* fermented product (BLFP) on day 4 was incorporated at the rate of 5, 9 and 12% respectively as a feed ingredient in juvenile *Penaeus monodon* diet based on their amino acid profile, proximate composition and selected mineral profile. The growth and feed utilization efficiencies of shrimp fed with fermented vegetable product were superior to those fed with diets containing unfermented product (UFP). Among the test diets, diet with 9 and 12% BLFP performed better in terms of specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), apparent net protein utilization (ANPU) and weight gain. The highest protein and fat digestibility of 57.36 and 74.32% respectively, were recorded in shrimp fed with diet containing 9% BLFP. The highest protein and fat deposition was for the diet with 9% (68%) and 5% (6.47%) incorporation of BLFP, respectively. From the present study, it has been concluded that 9-12% of BLFP can be supplemented in the diet for *P. monodon* for its better growth and survival.

Keywords: *Bacillus licheniformis*, Co-fermentation, Shrimp feed, Vegetable discard

Introduction

Fruit and vegetable markets produce a large amount of waste and its disposal costs a lot, both financially and environmentally. It has been proven that this waste can be upgraded to products of higher value that have a place in the market by utilizing its physico-chemical properties. Over the last years, considerable emphasis has been put on the recovery, recycling and upgrading of organic waste that can be transformed into several different products like bio-fuels, multifunctional food ingredients, nutrients, food flavours, fodder, feed and operational supply like bio-adsorbents for waste water treatment (Laufenberg *et al.*, 2003). Solid state fermentation is defined as any fermentation process performed on a non-soluble material that acts both as physical support and as a source of nutrients in the absence of free flowing liquid (Pandey, 1992). The important bacterial strains used for fermentation are *Bacillus* sp*., Pseudomonas* sp*., Serratia* sp*., Streptococcus* sp*., Lactobacillus* sp. and *Clostridium* sp. (Raimbault, 1998). *Bacillus* enzymes are very efficient in breaking down a large variety of carbohydrates, lipids and proteins into smaller units. *Bacillus* sp. grows efficiently with very low-cost carbon and nitrogen sources (Sonnenschein *et al*., 1993). It also degrades organic accumulation in ponds of shrimp cultures (Lin, 1995; Rengpipat *et al*., 1998; Verschuere *et al*., 2000). *Bacillus licheniformis* has been used in the fermentation industry for production of proteases, amylases, xylanase, antibiotics, and special chemicals for over a decade with no known reports of adverse effects to human health or the environment (Archana and Sathyanarayanana, 1997). The objective of the present study was to find the suitability of fermented vegetables discard as a feed ingredient for juvenile black tiger shrimp *Penaeus monodon* diet.

Materials and methods

Solid state fermentation

Vegetable discards collected from Ernakulam main market (Kerala, India) were used as the raw material for fermentation. Major discards in this market were banana peel and pieces, cabbage leaves, cauliflower leaves *etc*. Dried and powdered vegetable discard powder, soybean and wheat flour, mixed in the ratio of 0.5:1:0.5, were used as the substrate for fermentation. For the present study, 5 ml of 24 h - old culture of *Bacillus licheniformis* MTCC 6824, containing 10^7-10^8 cells ml^{-1} was aseptically inoculated into 500 ml conical flask containing 50 g sterile ingredient mixture adjusted to a final moisture of 50%. After mixing well, it was incubated at 37 ± 1 ^{0}C under static condition for 5 days. Triplicates were maintained and the samples drawn daily from every treatment were dried at 60 ºC in a hot air oven to obtain a constant dry matter weight. Dried samples were ground using a domestic mixer and stored in an airtight plastic container in a domestic freezer at -20 ^{0}C until further use.

Chemical analysis

Proximate composition of both unfermented and fermented vegetable powder were analysed following standard methods (AOAC, 1990). All chemical analyses were done in triplicates. Crude protein in the samples (0.5 g each) was estimated using Kjeltec 2300 (FOSS Tecator) unit. Crude fat in samples (2-3 g each) was determined using Soxtec ™ System 2043 extraction Unit (FOSS Tecator) and crude fibre (0.8 g sample) was determined as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH in Fibertec™ 2010 System (FOSS Tecator) and ashing at 550 ºC in a muffle furnace to a constant weight. Crude ash was determined by incinerating 3 g samples in a muffle furnace at 550 ºC for 3h. The crude ash thus obtained was digested with concentrated HCl, filtered and the residue was ignited at 600 ºC for 3 h, cooled and weighed to obtain acid insoluble ash (AOAC, 1990). Nitrogen Free Extract (NFE) was computed by taking the sum of values for crude protein, crude lipid, crude fibre and moisture and subtracting this value from 100 (Maynard *et al.*, 1979).

Amino acid analysis was carried out using acid hydrolysed (6 N HCl) samples by reverse-phase high-performance liquid chromatography (HPLC) after pre-column derivatization by phenyl isothiocyanate (PITC) by a modified method adapted from Fierabracci *et al.* (1991). HPLC was performed using Waters 1525 Binary HPLC pump and Waters 2487 Dual Absorbance Detector. Data were processed and analyzed using Waters Breeze software. Operating conditions were: column temperature - 38 ºC, column - pico-tag (waters, pico-tag system); absorbance - 254 nm; pump pressure - 1500– 1700 psi. Tryptophan content in the samples was determined by spectrophotometry after alkaline hydrolysis (5% NaOH). Absorbance value was measured at 500 nm (Genesis UV 10 Spectrophotometer) and the concentration was calculated from standard values and expressed as g 100 g^{-1} sample.

Minerals (magnesium, manganese, iron, zinc and copper) were analysed using atomic absorption spectrophotometer Analyst 300 (Perkin Elmer, USA) with air-acetylene flame (AOAC, 2006) and phosphorus was estimated by titrimetric method as per AOAC (2006). Phytic acid was estimated by the modified method described by Wheeler and Ferrel (1971) using thermospectronic spectrophotometer (UV-VIS Genesys 10uv) at 480 nm.

Feeding trial using juvenile Penaeus monodon

Bacillus licheniformis fermented vegetable product (BLFP) was evaluated in *P. monodon* diet during a 45 day feeding trial. Based on its amino acid profile, proximate composition and mineral content, BLFP of day 4 was

selected as an ingredient for shrimp diet preparation. Three diets were formulated by incorporating 5, 9 and 12% of BFLP. A diet containing 9% unfermented vegetable product (UFP), and prepared by incorporating 50% animal protein (clam meal, fish meal and shrimp head meal mixed in equal proportions) devoid of fermented ingredient (NCF) and one commercial diet (CF) served as control diets. The crude protein content was adjusted to 40 ± 2% while feed formulation. The feeds were extruded at 65 ºC in a twin-screw extruder using 2 mm die.

Penaeus monodon juveniles (average initial size 6 ± 0.25 cm, 1.5 ± 0.07 g) were procured from a farm at Chellanam, Kochi, India. Each treatment consisted of 3 replicate groups of 10 shrimps per tank along with control groups. Shrimps were fed at the rate of 10% of biomass twice daily by dividing the daily ration into two meals, fed at 09:00 and 15:00 hrs. The faecal matter was collected in to a collection sieve made of bolting silk (40 μ) without trapping the uneaten feed (Sudaryono *et al.*, 1996) from day 7 of commencement of the feeding trial. Fecal matter of the same treatment was pooled, gently rinsed and dried in an oven at 50 ºC for a day and stored at -20 ºC for further analysis. Apparent digestibility of nutrients (protein and fat) was determined for diets based on relative change in chromic oxide percentage in feed and faeces. The water quality parameters like salinity, temperature, dissolved oxygen, pH, ammonia- nitrogen, nitrite-nitrogen *etc.* were analysed every fortnight and maintained well within the permissible limits.

Diet performance

Diet performance was evaluated by calculation of weight gain, survival rate, feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), apparent net protein utilization (ANPU), apparent nutrient digestibility and apparent dry matter digestibility coefficient and body composition (Steffens, 1989).

Statistical Analyses

Results were statistically analyzed using one-way ANOVA. Post hoc tests like Duncan multiple range test (DMRT) (Duncan, 1955) and least significant differences (LSD) were conducted to find out whether variations existed between the treatments. Statistical significance was determined by setting aggregate type I error at 5% ($p < 0.05$) for each set of comparisons. All the analyses were done using SYSTAT version 7 and Microsoft excel.

Results and discussion

Vegetable discards appear to be a favourable substrate for SSF, as it is available at low cost in bulk quantities in tropical and subtropical countries and contains high cellulose content. In the present study, about 40% of the vegetables collected consisted of cabbage and cauliflower

leaves, 30% banana pieces, 5% beans and balance 25% of mixed vegetables. Substrate particles of 400 μ size were used in the present study based on report that 400 μ supported maximal cellulase production during fermentation process (Krishna, 1999).

Moisture content is the key parameter for regulating and optimizing SSF processes, since it influences microbial growth, enzyme activity, accessibility of the substrate and also regulates product formation (Narahara *et al.*, 1982; Nigam and Singh, 1994; Pandey, 1994; Pandey *et al.*, 2000). In the present study, during the process of standardization of SSF, 50% moisture content was maintained in the substrate with distilled water, since it has been reported that very low moisture content (<50%) leads to poor microbial growth due to lower mass transfers, resulting in slower conversion of substrate to biomass (Pitt and Hocking, 1997). While, moisture content above 60% retards microbial growth due to substrate agglomeration, poor aeration and decreased available area for growth (Ramesh

and Lonsane, 1991). The incubation temperature is yet another factor regulating growth and enzyme synthesis (Krishna and Chandrasekaran, 1996). According to Penaloza *et al.* (1985), fermentation time and temperature as well as the initial moisture content of the substrate have proved to exert significant effect on total amino acid content of the final product. A study by Imelda-Joseph and Paulraj (2003) has shown 48 h as the optimum duration of SSF for substrate modification of soybean meal and 36 h for mixed oil cakes using *B. coagulans* (Imelda-Joseph *et al.*, 2008). The process of SSF using *B. licheniformis* was standardized for obtaining the optimum duration of fermentation for substrate enrichment. The duration selected for fermentation was 0, 24, 48, 72 and 96 h, since bacterial multiplication is faster compared to fungi. The bacterial fermented products derived after day 4 was found to be the best in protein and amino acid profile (Table 1 and 2).

In the present study, a significant ($p < 0.05$) increase in the moisture content of 3.35% was observed with progress

Table 1. Proximate composition of *Bacillus licheniformis* fermented product (BLFP) (on dry matter basis)

Days	Moisture (%)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Ash (%)	NFE (%)
0D	3.26±0.80	21.22±0.82	0.78±0.08	5.90±0.12	5.80±0.12	63.05±0.28
1D	4.77±2.48	21.32±1.86	0.68±0.03	5.50±0.14	4.70±0.10	63.24±0.03
2D	3.52±0.51	24.39±0.88	0.79±0.06	5.28±0.13	5.59±0.14	59.72±0.18
3D	6.61±0.91	23.53±1.12	0.73±0.08	5.32±0.10	5.63±1.12	58.20±0.052
4D	3.65±1.27	27.48±2.85	0.68±0.03	4.80±0.26	6.61±0.23	56.77±0.043

Table 2. Amino acid profile of *Bacillus licheniformis* fermented product (expressed in g 100 g^{-1} sample)

Amino acids	0D	1D	2D	3D	4D
Aspartic acid	1.66±0.090	1.71±0.008	2.00±0.127	2.00±0.012	2.30±0.009
Glutamic acid	3.60±0.156	3.60±0.016	4.13±0.237	4.14±0.015	4.44±0.014
Serine	0.95±0.067	0.95±0.003	1.16±0.012	1.23±0.022	1.30±0.002
Glycine	0.63±0.039	0.63±0.002	0.75±0.018	0.77±0.003	0.66±0.006
Histidine	0.36±0.013	0.36±0.019	0.44±0.014	0.45±0.013	0.42±0.003
Arginine	1.03±0.028	1.00±0.066	1.24±0.053	1.21±0.015	1.25±0.023
Threonine	0.69±0.083	0.69±0.045	0.85±0.068	0.91±0.021	0.92±0.002
Alanine	0.63±0.033	0.65±0.002	0.82±0.081	0.81±0.005	0.84±0.002
Proline	1.12±0.079	1.04±0.008	1.23±0.106	1.16±0.021	0.90±0.001
Tyrosine	0.41±0.024	0.42±0.004	0.50±0.024	0.51±0.006	0.47±0.012
Valine	0.66±0.044	0.67±0.002	0.80±0.010	0.85±0.006	0.88±0.007
Methionine	0.17±0.008	0.17±0.008	0.18±0.007	0.22±0.006	0.23±0.008
Cystine	0.15±0.007	0.15±0.004	0.12±0.091	0.19±0.005	0.14±0.004
Isoleucine	0.54±0.032	0.56±0.002	0.67±0.043	0.68±0.005	0.66±0.007
Leucine	1.17±0.070	1.20±0.006	1.46±0.089	1.47±0.020	1.43±0.015
Phenylalanine	0.78±0.053	0.78±0.002	0.97±0.103	0.95±0.009	0.88±0.000
Lysine	0.58±0.021	0.58±0.121	1.00±0.045	0.86±0.11	0.92±0.001
Tryptophan	0.42±0.11	0.47±0.21	0.51±0.121	0.54±0.23	0.57±0.124
TOTAL	15.57±0.88	15.65±0.02	18.83±1.24	18.95±0.19	19.18±0.10

of fermentation, which may be due to the microbial respiration to produce carbon dioxide and water (Chutmanop *et al.*, 2008). Improved crude protein (29.5%) content was recorded with increasing duration of post-inoculation. The possibility for increase in the crude protein may also result from production of ammonia, amines, amino acids and peptides due to autolysis during the process of bacterial fermentation (Haard *et al.*, 1985). From earlier reports, it has been observed that if suitable substrate and organism are selected by providing optimum moisture, pH *etc.*, nutritional profile of many substrates can be improved by SSF (Ramesh and Lonsane, 1990; Krishna and Chandrasekaran, 1996; Ashok, 2003). The crude fat content of the fermented product in the present study showed a decreasing trend. A similar result was reported by Imelda-Joseph *et al.* (2008) using *B. coagulans*. Reduction in crude fat during the course of fermentation is explained as assimilation of lipids from the substrate possibly for biomass production by the bacteria. Loss of lipid in palm kernel meal during SSF due to its conversion into fungal protein biomass was reported by Wing Keong *et al.* (2002). Crude fibre content showed an increasing pattern (18.6%) during the progress of fermentation. It may be due to the utilization of easily digestible soluble carbohydrates by the bacteria leaving the indigestible fibre content high as reported by Singh *et al.* (1990). Crude ash content in the present study showed a significant increase during the progress of fermentation. This can be explained by the loss of organic matter by the process of fermentation. NFE content showed a decrease of 11% at the end of the fermentation period in the present study. The result show that the available carbohydrate in the substrate decreased with increase in protein and fat content during SSF suggesting the bioconversion of carbohydrate in to microbial protein and other compounds. Easily metabolizable carbohydrates may result in the better growth of the bacteria along with the reduction in the enzyme formation. However, in complex source of starch, organism grows very slow with significant secretion of alpha amylase in the fermentation medium (Rama and Srivastav, 1995).

The quality of protein in any feed is assessed by its amino acid composition. Total amino acid concentration increased on day 4 of inoculation. Peak concentrations of amino acids were seen on day 3 and day 4 of inoculation, with maximum peaks observed on day 4 (23.2%). The increase in major amino acids recorded on day 4 were for lysine, aspartic acid, serine, alanine, valine, methionine and threonine in the descending order (Table 2). Methionine content showed an increase in the present study, which is an essential change, as methionine has been generally reported to be the most limiting essential amino acid in plant proteins (Aguirre *et al.*, 1976; Raimbault, 1981). Reduction in proline and cysteine values was observed in

the present study. The increase in amino acid content may be attributed to the hydrolysis of protein to amino acid fractions as well as synthesis by the bacteria (Espe *et al.*, 1989; Hassan, 2003). Peptides and amino acids become available through proteolytic activities. It has been demonstrated that during fermentation, certain amino acids are produced and the availability of vitamins are improved (Nout and Motarjemi, 1997). The increase shows that the carbohydrate utilization is closely proportional to protein production during fermentation. The reduction in amino acids may be due to the utilization of these amino acids for the production of enzymes and other organic compounds by the microbial strain (Imelda- Joseph *et al.*, 2008). A comparison of the total free amino acid and fatty acid profiles of raw and fermented grass pea meal showed an increase in their availability in the fermented form.

According to Tacon (1987), minerals like calcium, phosphorus, magnesium, potassium, iron, zinc, copper, iodine and selenium are essential to shrimps for growth as well as various cellular activities. In the present study, variations in minerals like copper, manganese, zinc, magnesium, iron and phosphorus during the course of fermentation was analysed. Phosphorus (35.89%), copper (21.17%), zinc (31.53%), manganese (15.46%) and magnesium (21.33%) showed an increase in concentration whereas, iron showed a decrease in concentration during fermentation. (Fig. 1). The increase in the mineral content may be due to the reduction in phytic acid which is responsible for the binding of bivalent cations like iron, zinc, calcium and magnesium and decreasing their bioavailability (Ford *et al.,* 1978; Zyla, 1992; Raghuvanshi *et al.*, 2001).

The phytic acid content showed a reduction during the course of fermentation (33.33%) in the present study (Fig. 1), which may be attributed to the production of phytase by the microorganisms used in the fermentation process. Coffee pulp treated with *Bacillus* spp. showed a significant reduction on its anti-nutritional factors and additionally an increase in its protein content (Ulloa *et al.*, 2003). It is also reported to reduce tannins, phytates and
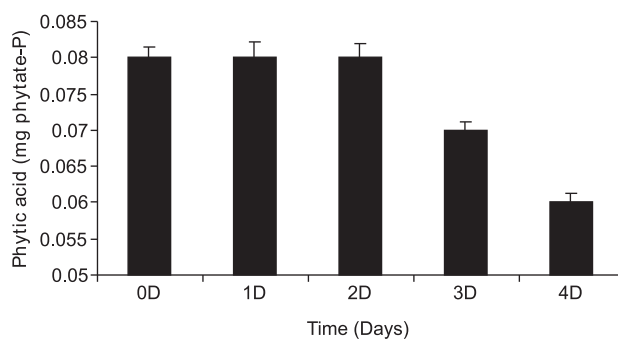

Fig. 1. Phytic acid content of *B. licheniformes* fermented product

mimosine to minimal values in leaf meals of *Lemna polyrhiza*, *Leucaena leucocephala* and *Lathyrus* seed meal after solid state fermentation (Bairagi *et al*., 2002; Bairagi *et al.*, 2004; Ramachandran *et al*., 2005).

Feed evaluation using P. monodon juveniles

Calorific value of all diets was above 3240 cal g^{-1}. According to Chen (1993), successful shrimp diets generally have to contain energy levels not less than 3200 kcal kg^{-1} diet. In addition to dietary energy level, the relationship between protein and energy in the diet is an important factor affecting growth response. The proximate compositions of the diets are presented in Table 3. The crude protein content of all the diets was adjusted to 40±2%. Among the fermented product incorporated diets, diets with 9-12% BLFP performed better in terms of SGR, FCR, PER, ANPU and weight gain (Table 4). The concept that carbohydrate can substitute proteins as energy source in shrimp feed is documented by Cruz- Suarez *et al*. (1994). Though some studies supported the use of carbohydrate, many authors have demonstrated that marine shrimps have severe restrictions for the utilization of dietary carbohydrate (Shiau and Peng, 1992; Le Chevalier *et al*., 2000).

Protein and fat digestibility of the diets in the present study was determined using the chromic oxide marker method which is accepted as a digestibility marker for *Penaeid* shrimp (Akiyama *et al*., 1989). Protein and fat digestibility reduced as the percentage of incorporation BFLP increased. The highest protein and fat digestibility

was reported in the shrimp fed with diet containing 9% BLFP (57.36 and 74.32%, respectively). The process of SSF itself would have modified the ingredients to easily digestible components with the release of enzymes that remain in the fermented product (Narahara *et al*., 1982; Pandey, 1992; Babu and Satyanarayana, 1996; Archana and Satyanarayana, 1997; Chutmanop *et al*., 2008; Rajmalwar and Dabholkar, 2009). Increase in weight gain showed a comparable relation with digestibility. According to Cruz-Suarez *et al*. (2001), when alternate plant protein sources are used in diets containing the same concentration of digestible energy and protein and are able to meet the nutritional requirements of the animal being fed, similar performance may be expected. The growth performance and feed utilization efficiency in fish fed fermented plant meal incorporated diet, performed better than those fed unfermented diets (Mukhopadhyay and Ray 1999; Bairagi *et al*, 2002, 2004; Ramachandran *et al*., 2005). The deposition of protein and lipid in the shrimp carcass at the termination of the feeding trial increased over the initial value in all dietary treatments. These results conform to the reports, where similar trends were noted with higher levels of inclusion of fermented sesame seed and leaf meals in carp diets (Mukhopadhyay and Ray, 1999; Bairagi *et al*., 2002, 2004). The protein and fat deposition was highest for 9% (68%) and 5% (6.47%) incorporation of BLFP, respectively. Dry matter percentage was also highest in diet with 5% BLFP. Similar observations were reported by previous studies also. Catacutan *et al*. (2003) studied

Table 3. Proximate composition (% dry weight) of test diets

Treatments	Moisture (%)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Ash (%)	NFE (%)
CF	2.36±0.65	41.84±0.28	6.37±0.02	5.62±0.32	13.25±0.15	30.56±0.15
NCF	2.38±0.25	42.57±0.02	5.65±0.05	6.34±0.25	15.68±1.25	27.38±0.21
UFP	2.14±0.65	40.74±0.25	5.52±0.36	6.66±0.6	15.89±0.35	29.05±0.86
BLFP1	1.89±0.35	41.24±0..33	5.30±0.59	5.50±0.15	14.35±0.56	31.72±0.4
BLFP2	1.67±0.36	41.41±0.32	6.50±0.36	5.50±0.25	14.52±0.54	30.40±0.55
BLFP3	1.58±0.21	40.98±0.25	5.87±0.36	5.79±0.56	14.61±0.18	31.17±0.25

NFE, Nitrogen free extract

Table 4. Growth performances and feed utilization efficiencies in *P. monodon* juveniles after 45 days feeding trial

Diets	Initial mean weight (g)	Final mean weight (g)	Weight gain (%)	SGR (%)	FCR	PER	ANPU (%	Survival (%)
CF	1.40	3.97	182.97	5.70	5.54	0.33	48.73	90
NCF	1.42	3.67	158.45	5.00	5.73	0.26	48.84	100
UFP	1.44	3.51	140.75	4.60	6.38	0.24	34.47	100
BLFP1	1.62	3.92	141.98	5.11	6.58	0.23	38.75	85
BLFP2	1.43	3.72	160.14	5.09	6.36	0.23	79.24	80
BLFP3	1.49	3.77	153.02	5.07	6.19	0.24	54.58	100

SGR = Specific Growth Rate; FCR = Food Conversion Ratio; PER = Protein Efficiency Ratio;
ANPU = Apparent Net Protein Utilization

the inclusion with various carbohydrate levels on the growth response of *P. monodon* and found that carcass crude protein showed no variation among treatments. Imelda-Joseph and Paul Raj (2007) had reported maximum carcass protein and lipid in the diet containing 35% substitution of *B. coagulans* fermented soybean in *P. monodon* post-larval diet. Carcass protein and lipid deposition was recorded maximum in the diet containing 30% fermented leaf meal for rohu fingerlings (Bairagi *et al.*, 2002).

In the present investigation, even though the fermented vegetable product incorporated diet was not better than commercial diet in growth performance, it is concluded that it can contribute to a certain extent in the shrimp feed industry, for their high nutrient content and cost effectiveness. Added advantage of reducing environmental pollution due to the improper disposal of the same can also be attributed by utilizing vegetable discards in a befitting manner. The product can be promoted as a minor ingredient or as an additive in the diet for *P. monodon* without compromising on growth. From the present study, an inclusion level of 9-12% BLFP in the diet for *P. monodon* has been recommended for commercial use and normal growth performance.

Acknowledgements

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