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Preservation of Phytase Enzyme Produced by Anaerobic Rumen Bacteria, *Mitsuokella jalaludinii*

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ABSTRACT

Poultry feed consists of feed ingredients like soybean meal and corn, which contain high levels of phytate that is poorly utilised especially by the monogastric animals that lack of phytase. Hence, phytase has been extensively applied as a feed supplement in poultry production due to the efficiency of this enzyme in improving phosphorous (P) availability, thus reducing P excretion to the environment as well as reducing the feed cost by reducing inorganic P supplementation. Mitsuokella jalaludinii, an obligate anaerobe, Gram-negative rumen bacterium, produces high phytase activity. Birds supplemented with bacterial preparation of M. jalaludinii showed comparable performance to that of commercial phytase. However, the anaerobic nature of this bacterium renders difficulty in the use of live cells as feed supplement in commercial poultry production. Therefore, this study was conducted to determine a suitable method to preserve phytase activity of M. jalaludinii regardless of cells viability. Mitsuokella jalaludinii was grown in MF medium under anaerobic condition and the cells were subjected to various treatments to preserve the enzyme, including bead beating, compressed air, moist heat, dry heat and freezedrying under aerobic condition. The results showed that the total number of viable cells were significantly (p<0.05) reduced when the cells were subjected to bead beating, whereas no viable cells were detected for compressed air, moist heat, dry heat and freeze-drying. Bead beating, compressed air, moist heat and dry heat treatments resulted in the reduction of phytase activity. However, only freeze-drying method was able to preserve high level of phytase activity significantly (p<0.05).

INTRODUCTION

Phytase is an enzyme that possesses phosphatase activity, which is able to hydrolyse phytate, the main form of bound phosphorus (P) predominantly present in cereal grains [1], to lower inositol phosphate derivatives and inorganic phosphate [2]. The existence of phytase was discovered a century ago in rice bran, however, it only become commercially available in the market as feed enzyme supplement in 1991[3]. Phytases drew the attention of both entrepreneurs and scientists for their ability to enhance the nutritional quality of plants materials in feed for monogastric animals by liberating phosphate [4]. Thus, the supplementation of phytase enzyme has become a regular practice in monogastric animal nutrition to overcome the anti-nutritive effect of phytate naturally present in the feed. Although there are diverse sources of phytase that have been characterised, microbial phytases are more acceptable for commercial production [5] and studies were focused more on fungal phytases. Over 200 fungal isolates in the genera *Aspergillus*, *Penicillium*, *Mucor*, and *Rhizopus* have been screened for phytase production [6,7]. The *Aspergillus* spp. have been recognized as the most active phytase producer [8,9]. *Aspergillus terreus* [10], *A. ficuum* [11], and *A. niger* [12] are species that are frequently observed to have phytase activity. Most of the microbial phytases that had been characterized exhibited the highest affinity to phytate among all phosphorylated compounds tested [13].

Recently, bacterial-derived phytases have become an alternative to fungal phytases due to several characteristics [14] such as wide pH profile, high thermal stability, phytate substrate specificity, and proteolysis resistance. Some examples of phytase producing bacteria are Bacillus subtilis [15,16], Pseudomonas [17], Klebsiella [18] and Escherichia coli [19]. Several rumen anaerobic bacteria like Megasphera elsdenii, Selenomonas ruminantium, and Mitsuokella multiacidus [20] were also reported to produce phytase enzyme. Although certain bacterial species such as those from Bacillus spp. [21,22] and Enterobacter spp. [23] produced extracellular phytase, but Escherichia coli was observed to produce phytase at the periplasmic space [24]. However, it can be generally concluded that the phytases produced by fungi are extracellular, whereas for bacteria, the enzyme is mostly cell associated including that of M. jalaludinii [25].

It was reported that phytase from *M. jalaludinii*, a strict Gram-negative anaerobic rumen bacterium was as effective as commercial phytase in improving broiler performance when freshly prepared *M. jalaludinii* was added to the feed [26]. The toxicity study carried out previously on rat [27] also verified the safety of phytase from *M. jalaludinii* for use in animal feed applications. However, it is not feasible to use freshly prepared cells for commercial application since the bacterial cells are anaerobic. Hence, the question arises whether the phytase from *M. jalaludinii* is still functional even when the cells became nonviable. None of the previous studies have reported on the methods for preserving anaerobic bacterial phytase. Therefore, in the present study, treatments to preserve phytase activity from *M. jalaludinii* were evaluated.

MATERIALS AND METHODS

Preparation of Bacterial Culture

Mitsuokella jalaludinii (DSM 13811^T; ATCC BAA-307^T) was grown in MF medium (modified M10 medium) [28] supplemented with 0.4% sodium phytate for 12 h at 39 °C under a strict anaerobic condition to harvest the cells for subsequent experiments. The culture was centrifuged at 10,000 × g for 10 min and washed twice with 0.1 M sodium acetate buffer (pH 5.0), after which the pellet was re-suspended in the same volume of the buffer. This was called the working cell suspension. One milliliter of the cell suspension was used to determine the initial colonies forming units (CFU) of cells by using the roll tube method [29] while another I mL of sample was used to determine the initial phytase activity. The rest of the cell suspension was subjected to different treatments.

Treatments of Mitsuokella jalaludinii

Mitsuokella. jalaludinii cells were subjected to various treatments that were selected based on the characteristics of the bacteria reported by Lan [30] and also the strict anaerobic nature of *M. jalaludinii*. Although these methods might cause cell death, but the main objective was to preserve the phytase activity. The active live cells were subjected to bead beating, compressed air, moist heat, oven dry heat and freeze-drying treatments. All samples were assayed for phytase activity and cells CFU at 1, 12 h, 18 h, 24 h, 30 h and 36 h after treatment, except for samples treated with lysis buffer and bead beating where analyses were conducted immediately after treatment.

Lysis Buffer and Bead Beating

This cell disruption method was conducted to break the cells. Two sets of tubes were prepared. One set was used to treat the cells with lysis buffer only, while the other set was further treated with bead beating method. The cell suspension was centrifuged and the cells were re-suspended in equal volume of lysis buffer (0.3 M acetate buffer; 25 M sucrose; 1 mM EDTA, 1 mg/mL lysozyme). It was shaken for 10 min at room temperature before transferring to the tubes pre-filled with 0.1 mm glass bead (MoBio Laboratories, Carlsbad, CA). The lysis method was performed according to the manufacturer protocol with slight modification. The samples were vortexed with maximum speed for 5 min. The bacterial cells together with the glass beads were centrifuged at 16,000 $\times g$ for 10 min. The supernatant containing the glass beads were discarded whereas the pellet was resuspended in equal volume of 0.1 M sodium acetate buffer (pH 5.0) and used to analyse for the phytase activity and cells CFU.

Compressed Air

Compressed air (Linde, Malaysia) was bubbled into the cell suspension in open serum bottle at a flow rate of 950 mL/min – 1000 mL/min for 4 h at room temperature. The treated sample was directly used for analyses.

Moist Heat

The cells suspension in test tubes sealed with butyl rubber stoppers were immersed in a shaking water bath set at 55 °C with an agitation speed of 180 rpm for 1 h and the samples were cooled in ice water for 15 min before the analyses.

Oven Dry Heat

The cell suspension was centrifuged at $10,000 \times g$ for 10 min to obtain the cell pellet and dried for 12 h in an oven which was preset to 45 °C. After the treatment, dried cells were kept in falcon tubes and placed at room temperature, re-suspended in equal volume of sodium acetate buffer (pH 5.0) before the analyses.

Freeze Dry

The freshly prepared cells (VOLUME)were kept at -20 °C for two and a half hours before frozen overnight at -80 °C. Samples were freeze dried (FreeZone[®] Freeze Dryer, Labconco, Kansas City, MO, US) for two days. The freeze-dried samples were placed at room temperature, re-suspended in equal volume of sodium acetate buffer (pH 5.0) before the analyses.

Phytase Enzyme Assay

The phytase enzyme assay [31] was conducted, in which the sodium acetate buffer (pH 5.0) was used as a replacement for the enzyme and substrate controls. Phosphorus was determined by using the ammonium molybdate method [32] and the amount of phosphomolybdate determined using was by spectrophotometer at the absorbance of 420 nm. One unit of phytase activity (U) was expressed as the amount of phytase enzyme needed to release one nanomol of inorganic phosphate per min per mL under the given assay condition. The culture was prepared in triplicates and the experiments were repeated three times. The active whole M. jalaludinii cells served as positive control for all the experiments conducted.

Statistical Analysis

One-way analysis of variance was conducted for all data using the General Linear Model (GLM) procedure of SPSS (Statistical package for the Social Science, version 22.0) software. Means were tested by the Duncan's New Multiple Range Test at 95%confidence level. All results were reported as means ± standard error.

RESULTS

Bead Beating

The results on phytase activity and CFU of cells treated with lysis buffer and by bead- beating with lysis buffer are shown in Table 1. Treatment by lysis buffer significantly (p<0.05) reduced phytase activity and cell growth compared to the control. Further reduction was observed in cells treated with bead beating and lysis buffer. The phytase activity of cells treated with lysis buffer was 192.56 ± 7.23 U and that with bead beating with lysis buffer was 148.11 ± 2.94 U. The cells CFU after treatment with bead beating with lysis buffer (3.46 Log CFU/mL) was significantly (p<0.05) lower than the other treatments.

Table 1. Phytase activity and growth of M. jalaludinii after lysis treatment.

Treatment	Phytase activity (U)	CFU (Log
		CFU/mL)
Control	255.15 ± 0.98^{a}	10.58 ± 0.002^{a}
Lysis buffer	192.56 ± 7.23 ^b	6.50 ± 0.002^{b}
Bead beating	148.11 ± 2.94°	$3.46 \pm 0.025^{\circ}$
with lysis buffer		

Values represent the mean ± SE of 9 replicates combined from total 3 experiments conducted a-c Means with different superscripts within the same column are significantly different (P<0.05)

Compressed Air

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The CFU decreased at every sampling time and after 30 h, no viable cells were detected (Fig. 1). A similar trend was observed for phytase activity, where the enzyme activity decreased at every sampling time. Significant (p<0.05) decreased in phytase activity was observed from 6 h onwards. At 36 h, phytase activity dropped from 73.17 ± 0.67 U (control) to 18.68 ± 1.26 U.



Fig. 1. Phytase activity versus growth of compressed air treated M. jalaludinii cells at different sampling times after treatment. a-d Bars with different letters are significantly different (P<0.05).

Moist Heat

The effect of moist heat on phytase activity and cells viability was rather drastic. As shows in Fig. 2, the viability of cells subjected to moist heat was significantly reduced (p<0.05) from 10.65 log CFU/mL to 3.3 log CFU/mL within 1 h, and viable cells were not detected at 12 h after treatment. Phytase activity decreased (p<0.05) significantly after the treatment and continued to decrease throughout the sampling times. The phytase activity dropped from 85.34 ± 0.76 U initially to $53.03 \pm$ 2.03 U at 36 h of sampling time.



Fig. 2. Phytase activity versus the growth of moist heat-treated M. jalaludinii cells at different sampling times after treatment. a-c Bars with different letters are significantly different (P<0.05).

Oven Dry

Fig. 3 shows the phytase activity and CFU of *M. jalaludinii* after being inactivated by oven drying method at 45 °C. No viable cells were detected after 1 h of oven drying, but phytase activity was still comparable to the control at 12 h sampling time (72.23 \pm 1.02 U). However, the activity started to decrease gradually but significantly (p<0.05) at longer sampling times.



Fig. 3. Phytase activity versus the growth of oven-dried M. jalaludinii cells at different sampling times after treatment. a-e Bars with different letters are significantly different (P<0.05).

Freeze Dry

The population of *M. jalaludinii* dropped drastically (p<0.05) after treatment from 10.90 Log CFU/mL (initial growth) to 4.74 CFU/mL (6 h), followed by 0.55 CFU/mL at 12 h (Fig. 4). Viable cells were not detected at 12 h onwards. Although viable cells were not detected, phytase activity was still present with the activity value comparable to the initial phytase activity.



Fig. 4. Phytase activity versus the growth of freeze-dried M. jalaludinii cells at different sampling times after treatment.

DISCUSSION

In the present study, several methods were evaluated for preserving phytase activity. This is an important consideration as the activity of phytase would be affected when the anaerobic M. jalaludinii cells applied as feed supplement, became non-viable when expose to the air. The viable cells were subjected to mechanical lysis with the intention to break the cell to determine whether the enzyme remained functional. Lysis by bead beating had been reported as the most effective lysis method [33] which is fast and suitable to apply for all range of bacteria [34]. The results obtained showed that application of either lysis buffer or the bead beating reduced the cell viability as well as resulted in the loss of phytase activity. Usually, a mixture of lysis method such as a combination of mechanical lysis and enzymatic lysis was applied for better lytic action. However, the present data (Table 1) showed that although this combined method reduced cell viability but the phytase activity was also affected at the same time. Vigorous beating of the cells would break the cell walls and since phytase was a cell-bound enzyme, the activity which is dependent on the conformational structure of the bound protein would be disturbed, hence causing the loss in activity. This showed that breaking up the M. jalaludinni cells was not an effective way in preserving the phytase enzyme.

Anaerobic bacteria cannot survive in the presence of oxygen as they lack enzymes such as superoxide dismutase, catalase and peroxidase that remove the toxic oxygen products [35]. Growth and phytase activity of *M. jalaludinii* cells were significantly affected (p<0.05) when they were exposed to the air. However, the cells were not killed immediately but the population of cells dropped gradually at every sampling times until the viability of the cells became undetectable at 30 h. The rate of cells reduction after exposure to the air depended on the level of sensitivity of bacteria to the oxygen [36]. Certain anaerobe would be killed immediately once exposure to the air whereas some could withstand the air for a certain period of time before the cells completely died.

A similar trend was noticed for both moist and dry heat in comparison with the compressed air to inactivate the whole cells where both the viability cell count and phytase activity were reduced after treatments. Both heat treatment methods were performed based on the previous report on the thermal stability of phytase enzyme from M. jalaludinii cells [25] as well as the temperature where cells were fully inactivated [30]. According to their findings, the growth of *M. jalaludinii* cells did not occur at 49 °C but for the phytase enzyme, 55-60 °C was the optimum temperature for the highest yield, and the activity declined drastically at temperature higher than 60 °C. However, the results from this study showed that phytase enzyme was not heat stable, and deteriorated with time. The changes in protein configuration due to denaturation caused by heat might be the reason contributed to the reduction of cells viability as well as the enzyme activity. The gradual loss in phytase activity might also probably due to the destructive activity of some other enzymes released by the cells when subjected to the stressful environment (oxygen and heat). From the observation, bacteria cells had to be killed immediately in order to retain the activity as the remaining population which slowly died under unfavorable condition tend to undergo autolysis. Bacteria will undergo autolysis due to several factors including growth phase, pH, temperature, osmotic pressure, the availability of oxygen as well as carbon and nitrogen sources [37].

This study showed that freeze-drying method to treat *M*. *jalaludinii* cells is a satisfactory approach to inactivate the cells

as well as to preserve the phytase activity. The inactivation effect of freeze-drying process toward *M. jalaludinii* cells was in accordance to the report by Strake and Stokes [38], where Gramnegative bacteria were more susceptible to a lower temperature (chilling, freezing, and cold shock) than Gram-positive bacteria. Although freeze-drying is widely used as a method to preserve and store bacteria in industrial application, but the process could be bactericidal due to the reduction of water activity of external environment that led to the accumulation of solutes in cells, resulting in osmotic stress [39]. Moreover, Teixeira et al. [40] also commented on the loss of viability of freeze-dried cultures, where cells were killed due to the result of oxidative degradation of membrane lipid as well as cell lesion. Although in the present study, *M. jalaludinii* cells became non-viable under freeze-drying treatment, but the phytase activity was well preserved.

The information on methods with bactericidal effect on *M. jalaludinii* is limited, but the results indicated that freeze-drying protected the cells from disruption and therefore maintained the enzyme configuration and hence its activity.

CONCLUSION

The viability of *M. jalaludinii* cells was not essential in order to retain the phytase activity, however, the cells must be inactivated with appropriate method. Among the methods evaluated (lysis buffer and beat beating, compressed air, moist heat, over dry, freeze dry), freeze-drying was the most suitable to preserve the phytase activity produced by anaerobic *M. jalaludinii*.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article

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