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Full Length Research Paper

Application of a new red carotenoid pigment-producing bacterium, *Enterobacter* sp. P_{41}, as feed supplement for chicken

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In order to use purple non-sulfur bacteria as feed supplement in chicken industries, screening for red pigment-producing bacteria with proteinases was done using hen feces as a target. One isolate, P_{41}, with the highest proteinases activity was selected for further studies. Based on the data of biochemical and 16S rDNA sequence analysis, it was identified as *Enterobacter* sp. P_{41}. This strain produced 0.16 mg of β-carotene with red color per gram of cell dry weight. Addition of the dried cells of *Enterobacter* sp. P_{41} to chicken feeds did significantly intensify the egg yolk color (p = 0.000). Moreover, addition of the dried cells at the amount of 0.45 mg per kg of feed significantly increased the rate of egg-laying (p = 0.029). These results suggest that the dried cells of *Enterobacter* sp. P_{41} might be useful as feed supplement for hens or other avian.

Key words: β-Carotene, *Enterobacter* sp., red carotenoid pigment, egg yolk, egg-laying rate, proteinase.

INTRODUCTION

β-Carotene, an organic compound with a red-orange color, is one of the most abundant carotenoids in many plants. It is a form of vitamin A which is necessary for both scotopic and color vision (Toomey and McGraw, 2011). Carotenoids are also important for the reproductive ability of female birds, as part of their fat soluble components including egg-yolk (Blount et al., 2002). Carotenoids can be found not only in plants, but also in microorganisms, especially in nearly all photosynthetic species (Marresca et al., 2007). *Rhodocyclus gelatinosus*, a purple non-sulfur bacterium, was used as a source of carotenoids for chickens to help intensify the red color in egg yolks (Kobayashi and Kobayashi, 1995). Chicken farmers in Thailand feed their chickens with carotenoids to intensify the color of their egg yolk. Most sources are from plants (Agricultural Academic Group, 1994). In previous times, they were not expensive but recently, chicken farmers have to face problems caused by seasonal and global warming effects that have resulted in significant higher costs of these plant derived materials. Photosynthetic bacteria might provide an alternative and cheaper source. They are able to grow in any seasons or places under controlled conditions. Moreover, the product yield can be increased by means of strain selection, mutation or optimization of growth conditions.

In addition, some photosynthetic bacteria are known to produce very active serine proteinases (Oda et al., 2004) which can digest proteins in feeds to release essential amino acids. In addition, a proteinase from *Lactobacillus delbrueckii* subsp. *bulgaricus* strains increased the growth of bifidobacteria by increasing the availability of the ami-

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no acids, valine, glycine and histidine (Saarela et al., 2000). Although the amount of protein did not influences egg production of hens significantly, the higher protein contents tend to have higher egg production than the lower protein content (Bunchasak et al., 2005). Thus, proteinase plays an important role in growth and egg production.

Based on this background information, we have screened for red pigment-producing non-sulfur bacteria producing proteinases in chicken feces. One isolate, P_{41}, identified as *Enterobacter* sp. P_{41}, was used to evaluate the effects of adding it to feed for hens in terms of the color level of the egg yolk and egg-laying rate.

MATERIALS AND METHODS

Glutamate-malate (GM) medium was used. It contains per liter 3.8 g sodium glutamate, 2.7 g DL-malic acid, 2.0 g yeast extract, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.8 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4.7H_2O$, 0.053 g $CaCl_2.2H_2O$, 1 mg nicotinic acid, 1 mg thiamine-HCl, 0.01 mg biotin, 1.2 mg $MnSO_4.5H_2O$, 1.0 mg $CoCl_2.6H_2O$, 2.5 mg ferric citrate. The pH was adjusted to 6.8 with 1 N NaOH. All purchased chemicals were of analytical grades.

Isolation of red pigmented or purple non-sulfur bacteria

Fresh feces of 50 hens obtained from the chicken-egg farm of the Department of Animal Science, Faculty of Natural Resources of the Prince of Songkla University, Hat Yai, was used to screen for purple non-sulfur bacteria or red pigmented bacteria. A total of 108 feces samples were collected separately from 5 to 6 month-old Hisex Brown hens. Bacteria were isolated by dilution and plate spreading methods. Serial dilution was done by weighing 1 g of the sample and transferring it subsequently to the test tubes containing 9 ml of 0.85% saline solution. The three dilutions (10^{-3}-10^{-6}) of the original sample were performed. Red pigmented, Gram-negative bacterial isolates growing on GM agar plates in a dessicator with 2/3 v/v of nitrogen gas added as under micro-aerobic conditions or conditions containing a lower level of oxygen than that of the atmosphere were selected as possible purple non-sulfur bacteria for further studies.

Cultivation of red pigmented or purple non-sulfur bacteria

Glutamate-malate (GM) medium was used for cultivating purple non-sulfur bacteria. A 13 ml of GM medium contained in a 15-ml screw-cap tube was inoculated with 1.5×10^8 cfu/ml of the Gram-negative red pigmented bacteria and incubated at 35°C for 48 h in the light (3,000 Lux), of Philips, a standard bulb, with compact size of 60 W. The culture broth was centrifuged at 12,000 rpm for 5 min. The supernatant was used to test for proteinase. The pellet of cells was dried at 60°C for 48 h or until no change in weight was used for further study.

Proteinase assay

Nutrient agar (NA) plates containing 1% skim milk at pH 8.0 were used to screen for extracellular proteinase-producing bacteria. Proteinase activity was measured by a modified Folin-Ciocalteau assay (Tanskul et al., 2009). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per ml of the reaction mixture per min.

Identification of selected isolates

Taxonomic characteristics of the isolate were identified according to Brenner and Farmer (Brenner and Farmer, 2005). In addition, 16S rDNA sequence analysis was carried out for further identification. Genomic DNA was extracted by using the standard method (Sambrook et al., 1989), and then amplified using the GeneAmp PCR System 9600. Universal primers were used; the F-primer was 5'-tggagagtttgatcctggctcag-3' and R-primer was 5'-taccgcggc-tgctggcacgtag-3'. The purified PCR products of the strains were in the range of 990 to 1500 bp. The amplified DNA was sequenced by using the ABI 377 DNA Sequencer (Applied Biosystems, Foster City, Calif.). The 16S rDNA sequence was compared with the NCBI database for the nearest equivalent identity.

Isolation of red methanol-acetone-chloroform-soluble pigment

The pigment of the dried cells was extracted directly into 3 mL of methanol, acetone, and chloroform each at room temperature using the method modified from Meckel and Kester (Meckel and Kester, 1980). The extract was then dried by evaporating, and kept for the determination of β-carotene.

Determination of β–carotene by reversed phase high performance liquid chromatography

The dried pigment containing residue was dissolved in absolute ethanol, and the solution was loaded onto a reversed phase high performance liquid chromatography column, Hypersil ODS 4.6 x 250 mm, 5 μm with an acetonitrile/methanol (10:90) solvent system at a flow rate of 1 ml/min at 30°C. β-carotene was quantified from the peak-height ratios by diode array detector from their absorbance at 450 nm (Wang et al., 2010). The external standard curve was linear in the range of 50 to 200 µg/ml.

Field experiments

9-month-old Hisex Brown hens were raised under optimal management conditions in the hen farm of the Animal Science Department, Faculty of Natural Resources, Prince of Songkla University, Hat Yai. The hens were randomly distributed to 5 treatment groups with 5 hens in each group.

Experimental feed preparation

A conventional feed for laying hens based on soybean meal (7 to 10%), dicalcium phosphate (0.2%), sodium chloride (0.5%), mixture of minerals and vitamins oil palm (3.0%), and exempt from antimicrobial agents was prepared (Agricultural Academic Group, 1994). Dried cells of *Enterobacter* sp. P_{41} were thoroughly mixed with the conventional feed at 0.15, 0.45 or 0.9 mg per kg and fed to the T3, T4 and T5 groups of laying hens, respectively. A positive control feed was prepared by mixing the conventional feed with 60% corn (a normal feed supplement) and fed to the positive control group of laying hens. For the negative control, feed corn was replaced with 60% broken-milled rice and fed to the negative control group.

Measurement of egg yolk color

The level of egg yolk color was measured visually by the DSM Yolk Color Fan, Dotterfarbfächer·Eventail colorimétrique Abanico

Table 1. Comparison of the characteristics of *Enterobacter* sp. and isolate P_{41}.

Characteristic	*Enterobacter* sp.[a]	isolate P_{41}
Gram staining	negative	negative
Cell shape	rod	rod
Motility	+	+
Pigmentation	yellow	red[b]
Photoautotrophic growth	ND	+
Citrate utilization (Simmons')	+	+
Urease	-	+
H_2S	-	-
Acid from sucrose	+	+
Acid from lactose	±	-
Indole test	-	-
Methyl red test	±	+
Voges-Proskauer test	±	-

[a], from Brenner and Farmer (2005); [b], in GM medium under micro-aerobic conditions with light, and on Tryptic Soy Agar (TSA) under light; +, positive; -, negative; ±, positive in some strains but negative in other strains; ND, not determined.

colorimétrico, a subjective method. The levels recorded for the color of egg yolk were on a color scale of 1 to 15, and from 1-light pale to 15-dark orange. Individual egg yolk color was measured daily throughout the experimental period.

Measurement of egg-laying rate

The rate of egg-laying of hens over a period of 5 weeks was measured by counting the number of eggs. Eggs were daily collected and egg production was calculated within a week. The average of the egg-laying rate was calculated on the last day of the week as number of egg per day.

Statistical analysis

All statistical analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA). The results abtained from five replications were statistically analyzed by factorial Anova and duncan's multiple range test (DMRT). Mean values were considered significantly different when $p < 0.05$.

RESULTS

Isolation of red pigmented bacteria

A total of 58 isolates of Gram-negative bacteria with a red pigmented colony were isolated from 108 samples from feces of 5 to 6 months-old chicken. Stock cultures were prepared in glycerol and kept at -80°C for further study.

Screening for bacteria producing extracellular proteinase

The casein Folin-Ciocalteau assay showed that 15 of the 58 isolates produced proteinase in the range of 0.2 to 0.8 unit. One of them, isolate P_{41}, gave the highest proteinase activity, of 0.8 units.

Biochemical and molecular identification of selected strains

The isolate P_{41}, that produced the highest proteinase activity, had taxonomic characteristics (Table 1) similar to those of an *Enterobacter* sp. (Gram staining, shape of cells, motility, citrate utilization, etc) except for its pigmentation and urease production (Brenner and Farmer, 2005). The 16S rDNA sequence of isolate P_{41} had a 100% similarity to that of *Enterobacter* sp. sb-3 (Accession no. EF152284) (Sun, 2006). Thus, isolate P_{41} was named as *Enterobacter* sp. P_{41}.

Determination of β-carotene by reversed phase high performance liquid chromatography

β -carotene from the isolated P_{41} cells was eluted from the column at 11.533 min. β-carotene was completely separated by using a Hypersil ODS column, and quantified from the peak areas detected by the diode array detector at 450 nm with an external standard. A calibration curve of β-carotene as external standard was used for calculating the amount of β-carotene based on peak area (data is not shown) and a control was used for estimating the retention time for the eluted β-carotene (Figure 1A). The isolated P_{41} cells contained 0.16 mg of β-carotene per g of cell dry weight (Figure 1B).

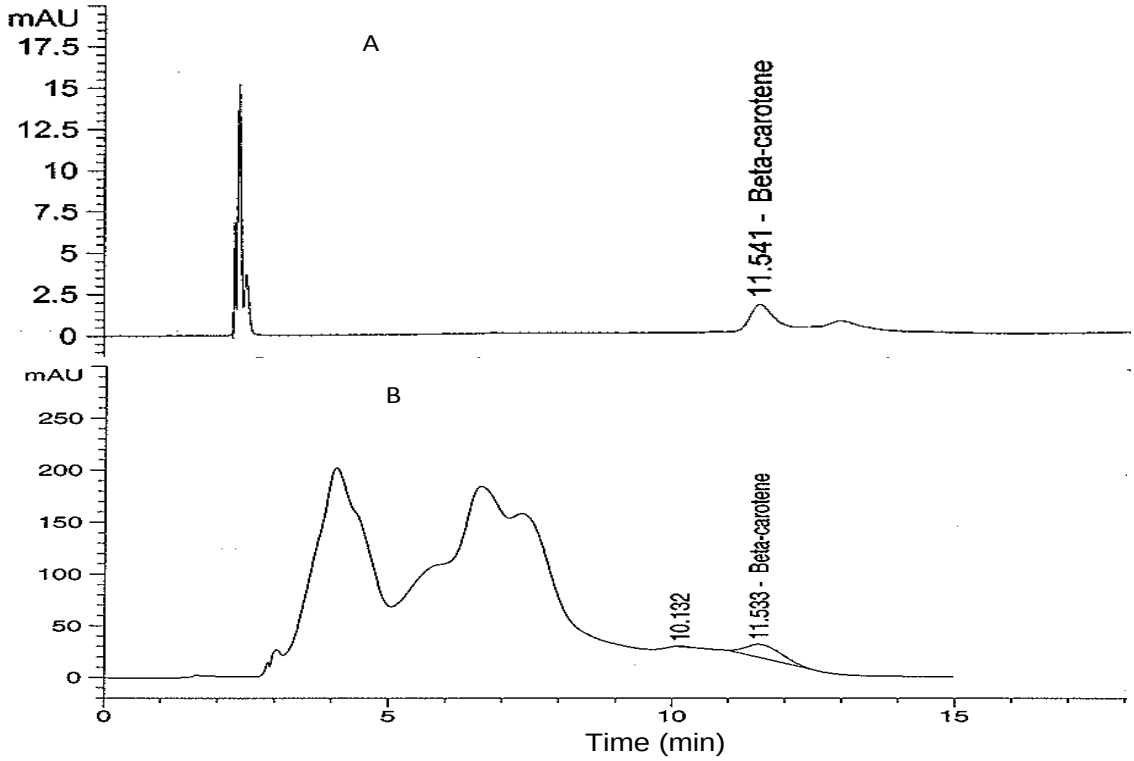

Figure 1. Reversed phase HPLC analysis of β-carotene extracted from *Enterobacter* sp. P_{41}. A, Control. B, Initial sample extracted from *Enterobacter* sp. P_{41}.

Effect of dried cells of *Enterobacter* sp. P_{41} on egg yolk color of hens

The individual egg yolk color of hens was significantly different (p = 0.000) between treatments during the observation period. From the first to the fifth week, the highest color level of egg yolk was recorded in the positive control group (T1) (from 8.524 $\pm$ 0.5118 to 9.595 $\pm$ 0.5617), whereas the lowest color level of egg yolk was observed in the negative control group (T2) (from 4.810 $\pm$ 2.6574 to 1.000 $\pm$ 0.000) (Table 2). The amount of color in T1 control was at least twice that in any of the eggs from hens treated with dried *Enterobacter* sp. P_{41} (Table 2).

Effect of dried cells of *Enterobacter* sp. P_{41} on the egg-laying rate of hens

For the egg-laying rate for T3, T4, T5, the positive and the negative control groups each was significantly different (p = 0.029). The egg-laying rate in T4 hens in each week, except the third week (4.86 $\pm$ 0.378) was the same as those of the positive control (T1 hens) (5.00 $\pm$ 0.000) (Table 2). In addition, the egg-laying rate for T3 and T5 hen groups in each week mostly was as the same as that of the negative control group (T2 hens) (Table 2).

DISCUSSION

Enterobacter bacteria are usually characterized by producing a yellow color on trypticase soy agar. However, *Enterobacter* sp. P_{41} isolated from chicken feces produced a red pigment on GM medium, and did not produce either a yellow or red pigment on trypticase soy agar (data not shown). β-Carotene present in the red pigment was identified by reversed phase high performance liquid chromatography. Other Enterobacteriaceae strains with carotenoid gene clusters were recently reported (Lehner et al., 2006). *Enterobacter sakazakii* is one of these isolates but its pigments are yellow (Lehner et al., 2006). There is no evidence that this isolate that is closely related to *Enterobacter* sp. P_{41} was pathogenic. Hence, *Enterobacter* sp. P_{41} was used as a feed supplement for hens. *Enterobacter* sp. P_{41} also produced an extracellular but heat labile proteinase, so perhaps it might be better added to the feed as a live source to improve the digestability of the proteins of the feed. Two of the other Gram-negative and red-pigmented bacteria without proteinase activity screened in this study, namely P_{12} and P_{72}, were identified as *Rhodopseudomonas palustris* NCIB8288 (Accession no. AF416661) and *Rhodopseudomonas palustris* DCP3 (Accession no. AF416663) (data not shown), respectively. These two strains might also be

Table 2. Color level of egg yolk and rate of egg-laying of hens for 5 weeks in each treatment. Mean values and standard errors corresponding to five independent experiments with five replicas each are represented.

Group	Week	Color level of egg yolk	Rate of egg-laying
T1	1	$8.524^{b} \pm 0.5118$	$5.00^{a} \pm 0.000$
	2	$9.333^{a} \pm 0.5774$	$5.00^{a} \pm 0.000$
	3	$9.333^{a} \pm 0.5553$	$5.00^{a} \pm 0.000$
	4	$9.452^{a} \pm 0.5221$	$5.00^{a} \pm 0.000$
	5	$9.595^{a} \pm 0.5617$	$5.00^{a} \pm 0.000$
T2	1	$4.810^{d} \pm 2.6574$	$4.14^{b} \pm 0.690$
	2	$1.810^{hi} \pm 0.6796$	$4.86^{a} \pm 0.378$
	3	$1.310^{ij} \pm 0.4323$	$5.00^{a} \pm 0.000$
	4	$1.214^{ij} \pm 0.4053$	$4.86^{a} \pm 0.690$
	5	$1.000^{j} \pm 0.0000$	$4.71^{a} \pm 0.488$
T3	1	$5.619^{c} \pm 2.3974$	$4.71^{a} \pm 0.488$
	2	$3.286^{ef} \pm 0.6437$	$4.71^{a} \pm 0.488$
	3	$2.738^{fg} \pm 0.6446$	$4.57^{a} \pm 0.535$
	4	$2.429^{gh} \pm 0.7295$	$4.86^{a} \pm 0.378$
	5	$1.929^{hi} \pm 0.6944$	$4.71^{a} \pm 0.488$
T4	1	$5.714^{c} \pm 2.1010$	$5.00^{a} \pm 0.000$
	2	$3.524^{e} \pm 0.8136$	$5.00^{a} \pm 0.000$
	3	$2.833^{efg} \pm 0.5986$	$4.86^{a} \pm 0.378$
	4	$2.738^{fg} \pm 0.6446$	$5.00^{a} \pm 0.000$
	5	$2.390^{gh} \pm 0.6648$	$5.00^{a} \pm 0.000$
T5	1	$5.238^{cd} \pm 2.0953$	$4.71^{a} \pm 0.756$
	2	$2.952^{efg} \pm 0.9207$	$4.86^{a} \pm 0.378$
	3	$2.905^{efg} \pm 0.6823$	$4.86^{a} \pm 0.378$
	4	$2.810^{efg} \pm 0.7822$	$4.71^{a} \pm 0.488$
	5	$2.190^{gh} \pm 0.5804$	$4.71^{a} \pm 0.488$

tested for use as an alternative source of carotene

The highest quantity of dried cells of *Enterobacter* sp. P_{41} in the diet did significantly intensify the egg yolk color higher than a value of the negative control. This was markedly in significant contrast to the positive control (T1) with corn. Na et al*.* (2004*)* reported that a high concentration of carotenoid supplements to feed lowered absorption efficiency (Na et al., 2004). Yolks of a more intense color may be required for specific markets, but the yolk color results from this experiment would be accepted by the commercial market. Maize is a common feed supplement for chickens used to produce an intense egg yolk color. Maize contains a majority of xanthophylls (oxidized carotenoids) (Moros et al., 2002) that help in the pigmentation of egg-yolks (Na et al., 2004). Moreover, the digestive stability of xanthophylls is significantly higher than that of β-carotene (Blanquet-Diot et al., 2009).

Addition of the dried cells at 0.45 mg per kg of laying hen feed (T4) increased the rate of egg-laying, to almost the same as those of the positive control. There was only one egg missed in one of five hens on the 17th day or in the third week of the experiment (data not shown). A higher amount of dried *Enterobacter* cells added to the feed (T5) reduced the rate (Table 2). These effects might be the results of a combination of several factors, but not the presence of the heat-labile proteinase from *Enterobacter* sp. P_{41}. Proteinase activity was abolished after drying at 60°C for 48 h (data not shown). The effect of proteinase from wet or growing cells of *Enterobacter* sp. P_{41} should be further investigated.

The dried cells of *Enterobacter* sp. P_{41} could allow the hens to maintain their egg-laying rate compared to the positive control and an increased rate when compared with the negative control. In addition, it could intensify the

yolk color with significant difference when compared with the negative control. Although *Enterobacter* sp. P_{41} obtained from hen feces produced β–carotene and could be used as a feed supplement in the form of dried cells for hens or other avian. Further research is required to optimize the optimal conditions of carotenoid extraction to get larger amount of carotene. The isolate in its living form might be supplemented to the feed to increase the yolk color, and egg-laying rate.

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