Journal of Biotechnology **14**(4): 761-768, 2016

CAROTENOID PRODUCING *BACILLUS AQUIMARIS* FOUND IN CHICKEN GASTROINTESTINAL TRACTS

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Received: 28.9.2016
Accepted: 30.12.2016

SUMMARY

Pigmented spore-forming bacterial strains were isolated from the gastrointestinal tracts of chickens for screening for heat-stable carotenoid-producing strains that could be applied as feed supplements. Of the seven heat-stable pigmented isolates screened, only two, yellow *Sporosarcina saromensis* CH1 and red-orange *Bacillus aquimaris* CH9, produced pigments with typical carotenoid absorbance peaks (400–500 nm). The CH9 carotenoids exhibited higher scavenging activity (73.2%) of DPPH free radicals than the CH1 carotenoids (35.9%) and carotenoids of the reference *B. indicus* HU36 strain (78.4%), in comparison to 100% activity of acid ascorbic at 18.75 µM as the standard. The CH9 strain produced high levels of carotenoids (439 µg [g DW]$^{-1}$) and formed nearly 100% spores, whereas the CH1 strain produced low levels of carotenoids (92 µg [g DW]$^{-1}$) and only achieved 30% sporulation. Chromatographic and spectral profiles of the carotenoids found in CH9 indicated the presence of as many as 11 different carotenoid types closely related to 1-HO-demethylspheroidene and keto/hydroxyl derivatives of γ carotene. We successfully produced concentrated orange CH9 spore powder at a high concentration of 6.1×10^{11} CFU g^{-1}; these spores were much more heat-stable (66% survival at 80°C for 20 min) than the reference *B. indicus* HU36 spores (9% survival at 50°C for 20 min). In conclusion, *B. aquimaris* CH9 is a promising probiotic carotenoid-producing strain, with heat-stable spores that should withstand the heat-treatment processing required for feed and food supplement production.

Keywords: *Bacillus, spores, carotenoids, heat-stable, gastrointestinal tract (GIT)*

INTRODUCTION

Bacillus species that produce carotenoids are attracting wide interests for potential applications in the pharmaceutical, food, and animal feed industries as colorants and health promoters. The beneficial effects of carotenoids on health are mediated by their antioxidant activity (Tyssandier *et al.*, 2004). In a number of studies, pigmented *Bacillus* species have been isolated and identified from soil, river, and pond sediments. These include a yellow-pigmented *B. safensis* (Fakhry *et al.*, 2008), a yellow-orange-pigmented *Bacillus indicus* (Suresh *et al.*, 2004), several pink-pigmented isolates of *B. firmus* (Pane *et al.*, 1996), and red-pigmented *B. atrophaeus* (Fritze, Pukall, 2001). In a study of pigmented *Bacillus* strains isolated from human faeces, the yellow-pigmented *B. indicus* HU36 has been most studied for its probiotic properties and high production of carotenoids (Hong *et al.*, 2008; Khajena *et al.*, 2010). The HU36 carotenoids have been identified more precisely as glycosyl-apolycopene and glycosyl-4′-methyl-apolycopenoate esters having C30–C40 residues, based on mass spectral analysis (Perez-

Fons *et al.*, 2011). Sy and colleagues have shown that the glycosyl carotenoids of HU36 show better of bio-accessibility and accumulation into plasma, liver and adipose tissue of rats than synthetic β-carotene (Sy *et al.*, 2012).

However, studies screening and characterising carotenoid-producing *Bacillus* sp. strains isolated from the gastro-intestinal tract (GIT) of livestock are lacking. In theory, GIT-isolated pigmented strains would have an advantage in colonising and surviving in the GIT of their host (Barbosa *et al.*, 2005), thereby creating friendly microflora with beneficial effects. In addition, no reports are available on the heat-stability of spores formed by carotenoid-producing *Bacillus* species, although heat-stability is an important criterion for probiotics for feed-pellet processing, which typically undergoes heat treatment at approximately 80°C-90°C for 1 min. Thus, we attempted to isolate pigmented *Bacillus* species that form heat-stable spores and produce carotenoids from the GIT of cage-free chickens, which are known to possess diverse microbiota, for application as feed supplements.

MATERIALS AND METHODS

Reference strains

B. indicus HU36 isolated from human faeces (a yellow-orange pigmented strain; Le *et al.*, 2006), non-pigmented strains including *B. subtilis* PY79 and *B. subtilis* HU58 isolated from human faeces (Tam *et al.*, 2006) were used as controls in the experiments described here.

Preparation of intestinal samples and isolation of pigmented strains

Eleven cage-free chickens were collected from 5 regions in northern Vietnam: Cao Bang, Ha Tay, Vinh Phuc, Phu Tho, and Thanh Hoa. Each chicken gastrointestinal tract (GIT) was prepared for collection of a mucosal suspension in 0.9% NaCl solution. Then the suspension was heated at 80°C for 20 min before plating on LB agar for incubation at 37°C for 24 h to obtain individual colonies.

General methods

Each pigmented isolate was grown in Difco Sporulation Medium (DSM) broth for 48 hrs and verified for sporulation efficiency by determining the titer of heat-resistant cells (65°C, 45 min) versus the total viable cell count (Nicholson, Setlow, 1990). Heat-stability of spores was measured by incubating 2×10^9 spores at various temperatures from 40 to 90°C for 20 min, followed by counting heat-resistant cells. Monitoring of the speed of bacterial growth was performed in Tryptic Soy Broth (TSB) at 37°C at various time points from 4 to 48 hrs. The shapes of bacteria and spores were observed under a transmission electron microscope JEM1010-JEOL (Hitachi). The other general methods to determine anaerobic growth, haemolysis, amylase and protease activities of isolates have been described previously (Hong *et al.*, 2008, Khajena *et al.*, 2009). To assign strains to bacterial species, 16S rRNA analysis was performed as previously described (Le *et al.*, 2006).

Pigment extraction and analysis

Pigment extraction and analysis from *Bacillus* isolates was performed following a previously reported method (Khaneja *et al.*, 2009). Two hundreds µL of extracts from 1.2 mg dry weight cells or 1 mL cultures were prepared to measure their absorbance values at OD_{450} (for the CH1 strain) or OD_{480} (for the CH9 strain). Concentrations of carotenoids in the extracts were determined from the standard curve of astaxanthin at concentrations from 0.625 to 10 µg/mL.

HPLC analysis was performed on a Shimadzu 20 AD-UFLC system consisting of a photodiode array (PDA) detector using a reverse-phase (RP) C_{30}, 5-µm column (250 mm × 4.6 mm i.d.) coupled to a C_{30} guard column (20 mm × 4.6 mm; Thermo). The mobile phase and elution conditions were identical to those described by Khaneja *et al.* (2009). The CH9 carotenoids were identified by comparison of spectral and chromatographic characteristics to those published for reference carotenoids (Britton *et al.*, 2004).

Production of pigmented spores

To induce spore production, *Bacillus* strains were cultured in DSM broth for 48 hrs in a fermenter (ANABIO R&D built in-house) at the optimal temperature (30–37°C) and pH (7.0-8.0) for each strain. The purification of spores has been described previously (Nicholson, Setlow, 1990). The purified spore suspensions were then spray-dried at 160°C and at an atomizer speed of 25,000 rpm (ANABIO R&D built in-house) for collection of spore powder.

Determination of DPPH antioxidant activity

DPPH antioxidant activity was measured

following a standard method (Sharma, Bhat, 2009). In brief, individual 1.5-mL extracts from approximately 0.5 g (wet weight) of vegetative cells were incubated with 500 µL of 250 µM 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma). The DPPH absorbance at 517 nm was measured before and after the reactions. In parallel experiments, 1.5 mL ascorbic acid (6.25-25 µM), was added as a quantitative standard.

RESULTS AND DISCUSSION

UV-VIS spectral wavelengths of CH1 and CH9 pigments

Of the total 110 spore-forming bacterial isolates which were found in intestine samples of eleven free-cage chickens, only 7 pigmented isolates remained stable after treatment at 80°C for 20 min. For screening of *Bacillus* producing carotenoids, we extracted pigments from vegetative cells of the 7 isolates for measurement of their absorbance profiles. Of the 7 pigmented isolates, only two strains produced pigments with UV/VIS spectral absorbance profiles between 350 and 550 nm. These included a yellow extract of the CH1 strain (Fig. 1B, upper image) which showed the highest peak 400 and 450 nm (Fig. 1A) and a orange-red extract of the CH9 strain (Fig. 1B, lower image) which showed the highest peaks at 400 and 480 nm (Fig. 1A). The remaining five extracts did not produce clear UV/VIS spectral profiles. These results were confirmed by measuring the negative UV/VIS absorbance obtained with PY79 and the positive UV/VIS absorbance, with 400- and 450-nm peaks, obtained with HU36 (Fig. 1A). Based on these data, the pigments isolated from the CH1 and CH9 strains from chicken GIT appeared to be carotenoids. We conducted further experiments to confirm their antioxidant activity.

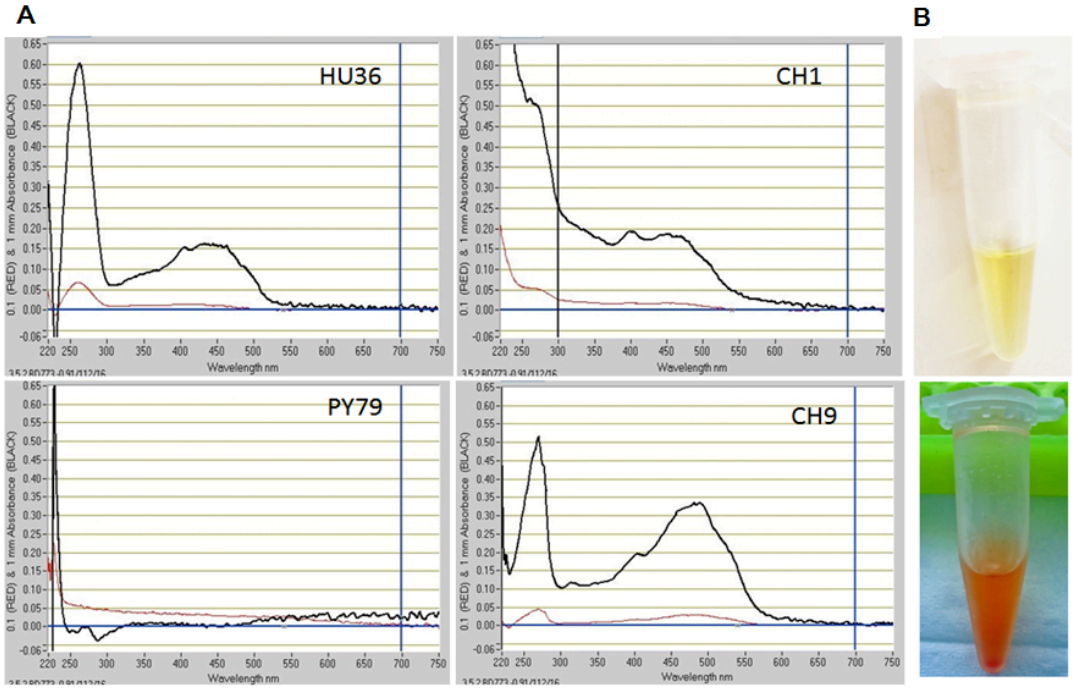

Figure 1. Panel A. Utraviolet visible (UV/VIS) absorbance spectra profiles from 220 nm to 750 nm of methanol-chloroform extracts of the yellow-pigmented *B. indicus* HU36 strain (positive control), non-pigmented *B. subtilis* PY79 (negative control), and the CH1 and CH9 strains isolated from chicken gastrointestinal tract. Panel B. Yellow and red-orange colours of extracts of the CH1 strain (upper panel B) and the CH9 strain (lower panel B).

Antioxidant activities of CH1 and CH9 pigments

An important criterion for identifying carotenoids is antioxidant activity. Thus, to confirm that the pigments found in CH1 and CH9 were carotenoids, we used a standard scavenging assay of

free DPPH radicals. As shown in Fig. 2, CH1 extract provided lower DPPH scavenging activity (35.9%) than that provided by CH9 (73.2%). As controls, ascorbic acid at a final concentration of 4.69 µM provided approximately 50% activity, HU36 extract provided 78.4% activity, and no scavenging activity was detected for the PY79 extract. Based on this data, we concluded that the pigments found in CH1 and CH9 vegetative cells showed the potential to be antioxidant carotenoids, and that carotenoids of the CH9 strain exhibited stronger antioxidant activity than those of the CH1 strain.

Identification of CH1 and CH9 strains

Because both CH1 and CH9 strains showed the potential to produce antioxidant carotenoids, they were further examined for their physiological properties (size of vegetative cells, spore position and growth conditions), their abilities to produce digestive enzymes (amylase and protease), and non-haemolysis. Representative images are shown in Fig. 3. Phenotypically, CH1 colonies were yellow and CH9 colonies were red-orange (Fig. 3A). CH1 formed nearly spherical spores (600 nm × 800 nm) at the terminal position whereas CH9 formed ellipsoidal spores (600 nm × 1000 nm) at a sub-terminal position (Fig. 3B). Both strains grew in aerobic, but not in anaerobic, conditions. In the starch hydrolysis test, CH9 showed stronger amylase activity than CH1. Casease activities were weak in both strains. No haemolysis zone was observed in both CH1 and CH9, indicating that they belong to the γ-hemolysin type. CH1 could form only 30% spores after 48 hrs of incubation in DSM broth, whereas CH9 could form almost 100% spores. Based on 16S rRNA sequencing data and BLAST analysis, the CH1 (GenBank accession no. KF432019.1) and CH9 (GenBank KM516787) strains were shown to be closely related to *Sporosarcina saromensis* (Similarity score: 0.99; Max score: 2488; E-value: 0.0) and *B. aquimaris* (Similarity score: 0.99, Max score: 2532; E-value: 0.0), respectively. We conclude that CH9 shows higher sporulation efficiency and amylase activity than CH1, which are important criteria for heat-stable probiotic strains.

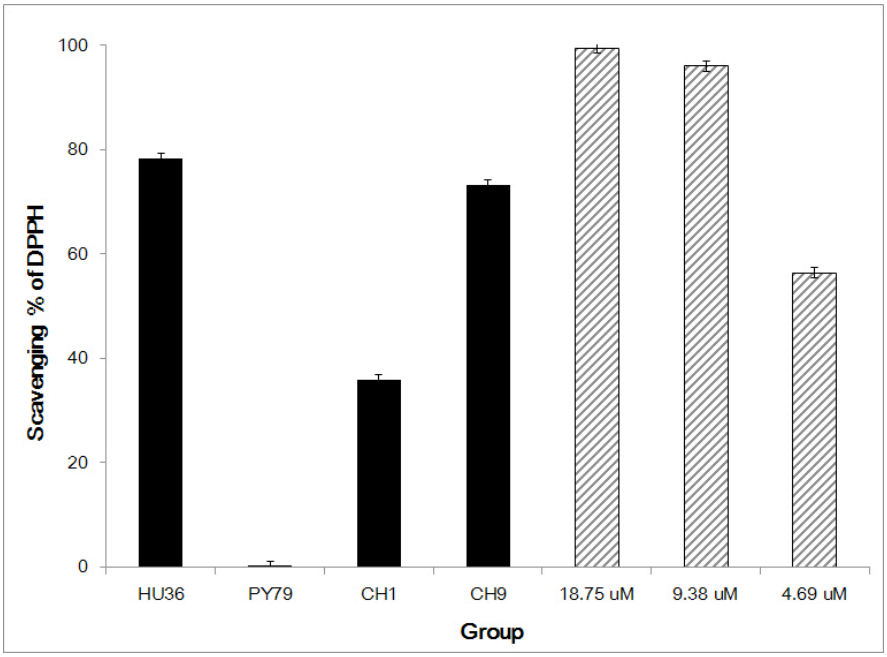

Figure 2. Scavenging percentages of DPPH free radicals (at a final concentration of 62.5 µM) by 1.5-mL extracts from 0.5 g (wet weight) cells of the *Bacillus indicus* HU36 strain (positive control), *B. subtilis* PY79 (negative control), the CH1 and CH9 strains and a standard antioxidant (ascorbic acid, diagonal bars) at final concentrations of 18.75 µM, 9.38 µM and 4.69 µM.

High level of antioxidant carotenoid production by the CH9 strain of *B. aquimaris*

We next compared carotenoid production levels between CH1 and CH9 strains by measuring the absorbance peaks (at 450 nm for CH1 and at 480 nm for CH9) of solvents extracted from CH1 and CH9 vegetative cells grown at various time points.

Absorbance at 600 nm at the same time points was also measured to monitor cell growth. As shown in Fig. 3C, both CH1 and CH9 strains grew quickly, reached the log phase after 4 h of seed culture (1:100 ratio), and reached maximum OD_{600} after 48 hrs. However, as indicated by changes in the OD_{450} values and the OD_{480} values, in both strains, carotenoid only reached detectable levels after 8 hrs of cultivation, reached the maximal level at 24 h, and then declined slightly at 48 h. The reduction in carotenoid production may have been caused by sporulation, which occurred from 24 h to 48 h. Pigment production of CH1 was approximately 6-fold lower than that of CH9 at 24 h, as indicated by the 6-fold lower OD_{450} of CH1 (0.104) compared to OD_{480} of CH9 (0.611). Based on the linear standard curve for the absorbance of astaxanthin (Fig. 3D), we calculated that the equivalent total carotenoid concentrations of the CH1 and CH9 extracts were approximately 0.73 and 3.7 µg/mL, respectively. These values correspond to a total carotenoid production quantity (µg) per milligram dry weight of cells (DW) of 92 µg $(DW)^{-1}$ for the CH1 strain and 439 µg $(DW)^{-1}$ for the CH9 strain. Because CH9 produced greater amounts of amylase and carotenoids, and showed higher antioxidant activity and sporulation efficiency, the CH9 strain would be the probiotic strain of choice for industrial production of carotenoid-containing feed supplements. Thus, we performed further characterisation of the carotenoid contents and spore properties of the CH9 strain.

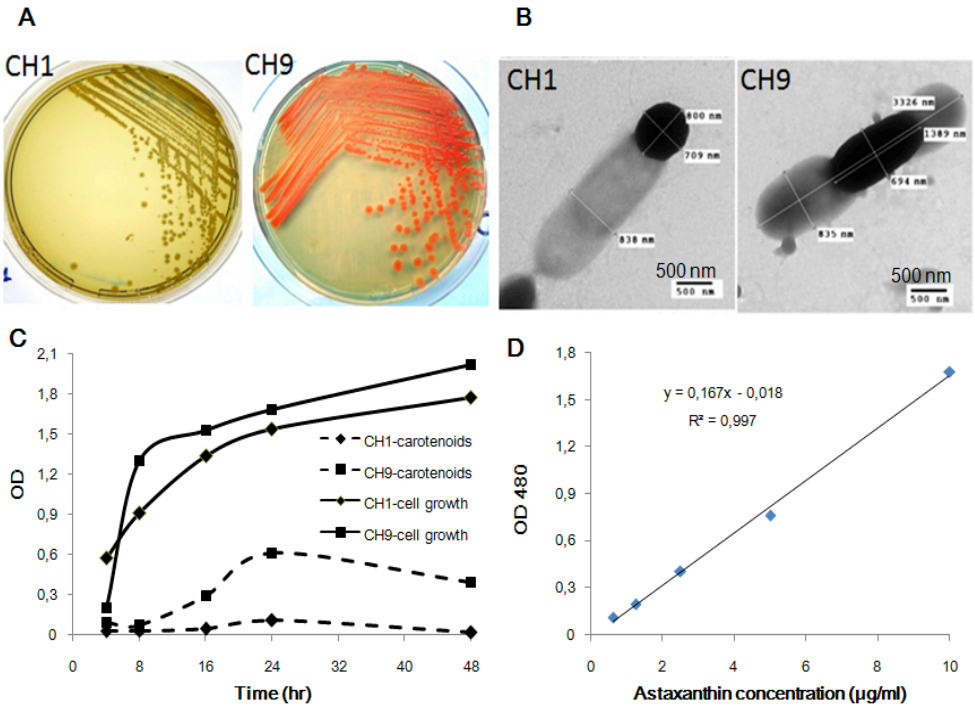

Figure 3. Panel A. Yellow and red-orange colonies of CH1 and CH9, respectively, grown on TSA medium. Panel B: Shapes and positions of spores of CH1 and CH9 within vegetative cells observed under a transmission electron microscope. Panel C. Bacterial growth and carotenoid production curves during cell culture in Tryptic Soy Broth. OD_{600} of bacterial cell cultures of CH1 (solid line, diamond) and CH9 (solid line, square) strains. OD_{450} and OD_{480} of 200 µl methanol-chloroform extracts from 1-mL cell cultures of CH1 (dash line, diamond) and CH9 (dash line, square), respectively. Panel D. Astaxanthin standard curve describing the correlation between OD_{480} and astaxanthin concentrations ranging from 0.625 to 10 µM (R^2 = 0.997).

Carotenoid profiling of CH9

To clarify the type of carotenoids produced by the CH9 strain, the carotenoid extract of CH9 was analysed by HPLC-PDA. Typical HPLC chromatographic profiles for the CH9 extract were recorded at 450 nm and are presented in Fig. 4. The physical characteristics of the carotenoids detected were concordant with the visible colour of the

orange-red CH9 colonies, as fractions showing absorbance peaks at 467–524 nm (orange, pink, red) were more abundant than fractions showing absorbance peaks at 450–456 nm (yellow). The absorbance spectra of the collected fractions with peaks at approximately 400–500 nm suggested that the CH9 strain produced at least 11 different carotenoid types. Comparison with reference spectra identified the carotenoids in the six fractions, including n°1–5 and n°11 as closely related to 1-HO-demethylspheroidene, and the carotenoids in the remaining five fractions, n°6–10, as closely related to keto/hydroxyl derivatives of γ carotene (Britton *et al.*, 2004, Le *et al.*, 2006).

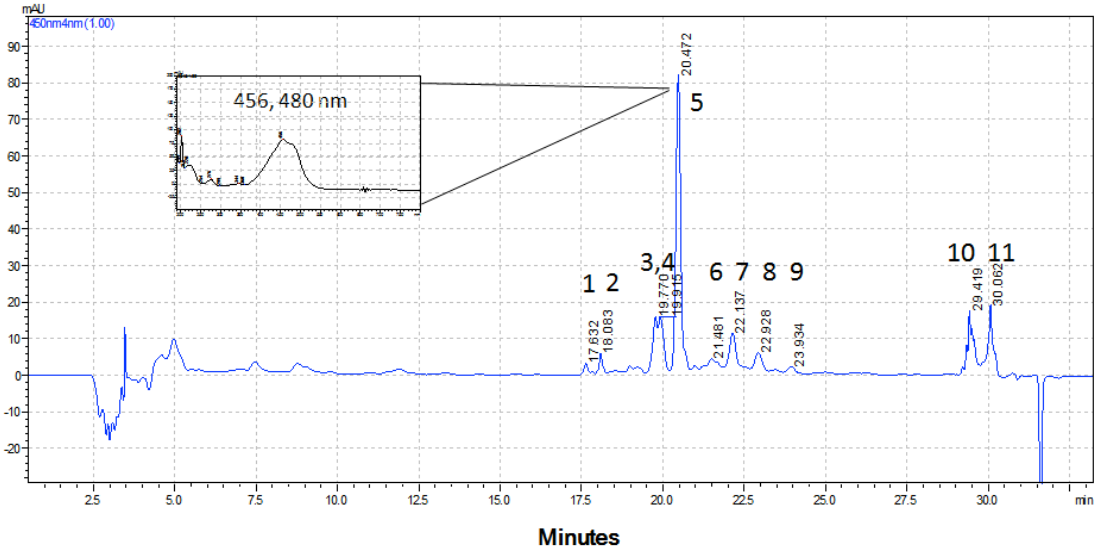

Figure 4. HPLC chromatograms with detection at 450 nm showing carotenoids in CH9 vegetative cells. λ max peak n°1 (455, 475); n°2 (455, 475); n°3 (450, 470); n°4 (450, 470); n°5 (456, 480); n°6 (467, 486, 513); n°7 (467, 490, 519); n°8 (467, 486, 515), n°9 (467, 486); n°10 (467, 490, 524); n°11 (454, 480). Highest peaks underlined.

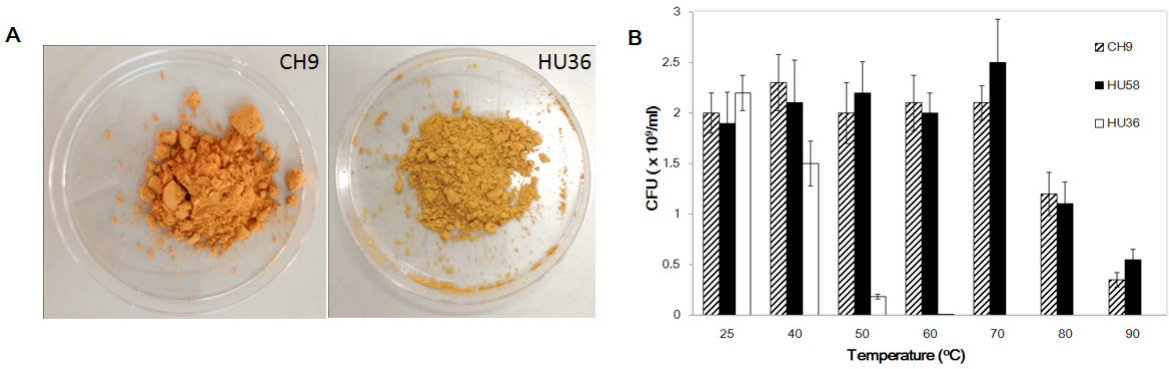

Figure 5. Panel A. Spray-dried powder of red-orange CH9 spores (6.1 × 10^{11} CFU g^{-1}) and yellow HU36 spores (1.1 × 10^{11} CFU g^{-1}). Panel B. Heat-stability of CH9, HU58, and HU36 spores. Heat-counts of CH9, HU58, and HU36 after treatment at various temperatures ranging from RT to 90°C for 20 min.

High sporulation yield of the CH9 strain and heat-stability of CH9 spores

Bacillus strains are thought to be able to easily form heat-stable spores. However, pigmented *Bacillus* strains do not always possess this ability, and our recent primary investigation of 15 pigmented *Bacillus* strains isolated from shrimp GIT indicated that strains differed with respect to their both sporulation efficiency and heat-stability of spores (data not

shown). To apply the CH9 strain in feed production, we needed to assess whether the CH9 spores are easily produced and heat-stable. For this purpose, we attempted to culture CH9 spores in DSM broth for 48 hrs using a fermenter; the resulting spore suspension was spray-dried to obtain a heat-stable spore powder. As a control, we also produced spore powders of the medium-sporulating HU36 strain (~50%) and the high-sporulating HU58 strain (almost 100%). As expected, we obtained red-orange CH9 spore powder at the very high concentration of 6.1×10^{11} CFU g^{-1} (Fig. 5A, left image). This concentration was almost equal to that obtained for HU58 spores (5.8×10^{11} CFU g^{-1}), and was 5-fold higher than that obtained for HU36 spores (1.1×10^{11} CFU g^{-1}; Fig. 5A, right image). To explain this 5-fold difference in spore concentration, the heat-stability of the CH9 spores was compared to that of HU36 and HU58 spores. As shown in Fig. 5B, HU36 spores were surprisingly heat-sensitive as the count was reduced to 8% at only 50°C for 20 min. In contrast, CH9 spores were stable at 80°C (60% survival for 20 min) and survival of 17.5% was maintained at 90°C. The heat-stability of CH9 spores were almost equal to that of the *B. subtilis* HU58 spores (55% survival at 80°C for 20 min, 23% survival at 90°C for 20 min); the heat-stability of this strain had been characterised for use as food ingredient (Permpoonpattana *et al.*, 2012). We did not obtain such strong heat-stability upon testing spores of the 15 strains isolated from shrimp GIT (50% survival at 50-70^{o}C for 20 min). Thus, for application in the feed industry, the CH9 strain should be superior to HU36, due to its high spore production and survival during feed processing, which normally requires heat treatment at approximately 80°C for 15–20 min.

CONCLUSION

To the best of our knowledge, this is the first study to isolate and characterise carotenoid-producing *Bacillus* strains from chicken GIT. The *B. aquimaris* CH9 strain was shown to possess several interesting properties, including (i) production of various antioxidant carotenoids at high levels (423 µg [g DW]$^{-1}$), (ii) high sporulation efficiency (100%, 6.1×10^{11} CFU g^{-1} spore powder) of heat-stable spores (60% survival at 80°C for 20 min). Thus, CH9 shows potential as a carotenoid-producing strain for further study of its probiotic activity *in-vivo* model in order to develop a novel feed and food supplements in future.

Acknowledgement: *This research is funded by Vietnam Ministry of Science and Technology and the Vietnam National University, Hanoi (VNU) under project number KLEPT.12.03 of Key Laboratory of Enzyme and Protein Technology and project number* 16-549 RG/BIO/AS_G – FR3240293311 of Third World Academy of Science (TWAS) *to Nguyen T. V. Anh. We thank Simon M. Cutting for the kind gift of the reference strains, Vu T. M. Duc, Tran T. My, and Nguyen T. N. Trang for technical assistance.*

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BACILLUS AQUIMARIS SINH TỔNG HỢP CAROTENOID PHÂN LẬP TỪ HỆ TIÊU HÓA CỦA GÀ

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TÓM TẮT

Các chủng vi khuẩn tạo bào tử có sắc tố được phân lập từ ruột gà để sàng lọc những chủng thuộc chi *Bacillus* bền nhiệt sinh tổng hợp carotenoid. Trong số 7 chủng đã sàng lọc, chỉ có 2 chủng có sắc tố *Sporosarcina saromensis* CH1 màu vàng và *Bacillus aquimaris* CH9 màu đỏ-da cam, sinh tổng hợp sắc tố với các đỉnh hấp thụ đặc trưng cho carotenoid (400–500 nm). Carotenoid của chủng CH9 có hoạt tính trung hòa gốc tự do DPPH (73.2%) cao hơn hoạt tính của carotenoid của chủng CH1 (35.9%) và của chủng tham chiếu *B. indicus* HU36 (78,4%), khi so sánh với 100% hoạt tính của chất chuẩn acid ascorbic ở nồng độ 18.75 μM. Chủng CH9 sinh tổng hợp carotenoid với hàm lượng cao nhất (439 μg [g DW]$^{-1}$) và tạo gần như 100% bào tử, trong khi chủng CH1 sinh tổng hợp carotenoid với hàm lượng thấp (92 μg [g DW]$^{-1}$) và chỉ đạt 30% tỷ lệ bào tử. Phổ sắc ký hấp thụ các carotenoid của chủng CH9 cho thấy sự có mặt của 11 loại carotenoid khác nhau có tính chất gần gũi với 1-HO-demethylspheroidene và dẫn xuất keto/hydroxyl của γ carotene. Chúng tôi đã sản xuất thành công bào tử CH9 dưới dạng bột sấy phun màu da cam ở nồng độ cao 6.1×10^{11} CFU g^{-1}; các bào tử này bền nhiệt hơn nhiều (66% sống sót ở 80°C trong 20 phút) so với bào tử của chủng tham chiếu *B. indicus* HU36 (8% sống sót ở 50°C trong 20 phút). Vì vậy, *B. aquimaris* CH9 là chủng vi khuẩn tiềm năng sinh tổng hợp carotenoid, tạo bào tử chịu nhiệt có khả năng duy trì độ sống khi xử lý nhiệt trong quá trình chế tạo thức ăn bổ sung.

Từ khoá: *Bacillus, bào tử, carotenoid, bền nhiệt, đường ruột*