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# STUDY ON BIOCHARACTERISTICS AND PURUVATE PRODUCTION OF MODERATELY HALOPHILIC BACTERIA ISOLATED FROM *Dunalliella tertiolecta*'s CULTURE MEDIUM

**Hoang Thi Lan Anh[1], Luu Thi Tam[1], Dang Diem Hong[1, 2*]**

[1]Institute of Biotechnology, VAST, Vietnam
[2]Graduate University of Science and Technology, VAST, Vietnam

## ABSRACT

Moderately halophilic bacteria designed strain D34 was isolated from the culture medium of green microalga *Dunaliella tertiolecta.* The isolate was Gram-negative, aerobic, rod-shaped, approximately 0.45–0.60 μm wide and 1.25–5.10 μm long, occurring singly, non-motile, and flagellum-less. Colonies on solid media are cream, circular, and smooth. This strain was able to produce exopolysaccharide, poly hydroxybutyrate, oxidase and catalase positive. Growth occurred in a temperature range of 20–40°C, a salt concentration of 0.1–25% (w/v), and pH range 6–12. The major fatty acids were $C_{16:0}$ (35.59%), $C_{16:1\omega-7}$ (20.54%), $C_{18:\ 1\omega-7}$ (30.14%), and $C_{12:0}$ (10.03% of total fatty acids). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain D34 belonged to the genus *Halomonas*. The highest levels of 16S rRNA gene sequence similarity were found between the strain D34 and *H. aquamarina* (sequence similarity 98.6%). Pyruvate, a central intermediate in metabolism processes in all organisms, is widely used for the synthesis of various chemicals and polymers as well as ingredient or additive in food, cosmetics, and pharmaceuticals. In this study, pyruvate production by strain D34 following changes in culture medium, glucose and nitrate concentrations and culture temperature were also studied. In 84 hours of batch cultivation, pyruvate production by wild-type *Halomonas* sp. D34 reached 37.24 g/L at 37°C with 20% glucose and 30 g/L sodium nitrate adding to SOT medium. These data provided evidences for pyruvate production using novel wild-type strains.

*Keywords: Halomonas*, moderately halophilic bacteria, organic acid, nitrate, pyruvate**.**

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[*]*Corresponding author email*: ddhong60vn@yahoo.com

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## INTRODUCTION

Vreeland et al. (1980) first proposed the genus *Halomonas*, which is the largest genus in the family Halomonadaceae, with *Halomonas elongata* as the type strain (Cao et al., 2013). At the time of writing, 102 species have been assigned to the genus *Halomonas* (https://www.bacterio.net/halomonas.html). *Halomonas* strains have been isolated from various saline habitats, such as salt lakes, marine environment, soils, salters, seafood or culture medium of microalgae (Cao et al., 2013; Yin et al., 2015). *Halomonas* species

are attracted attention for chemical production because of its simple medium composition and high chemical productivity such as polyhydroxyalkanoates, ectoine and hydroxyectoine, enzymes (amylase, protease, xylanase, and cellulase) owing to their unique, desirable properties as contamination-free culture conditions, wide temperature, pH ranges and high substrate concentrations (Kawata et al., 2012; Yin et al., 2015). Recently, Kawata et al. (2016) reported that wild type *Halomonas* sp. KM-1 could be produce pyruvate with productivity of 63.3 g/L at optimum conditions.

Pyruvic acid, an important oxocarboxylic acid, locating at the vital junction of cell metabolism, plays a central role in energy and carbon metabolism (Yonehara & Miyata, 1994). Currently, it is used mainly for the synthesis of various chemicals and polymers or as ingredient or additive in food, cosmetics, and pharmaceuticals (Li et al., 2001). Pyruvate can be produced by chemical synthesis (Ai & Ohdan, 1995), fermentation process (Li et al., 2001); enzymatic process (Eisenberg et al., 1997), and resting cells (Ogawa et al., 2001). On an industrial scale, pyruvate is produced by dehydration and decarboxylation of tartaric acid (Li et al., 2001). To date, the successful biotechnological production of pyruvate from glucose has primarily been achieved using metabolically engineered bacteria, such as *Escherichia coli* (Zhu et al., 2008) and *Corynebacterium glutamicum* (Wieschalka et al., 2012), and multi-auxotrophic yeasts such as *Saccharomyces cerevisiae* (van Maris et al. 2004) and *Torulopsis glabrata* (Liu et al., 2007). However, the difficulty of achieving genetically modified strains as well as complicated cultivating process are the reasons of the finding of novel, wild strains/species that can produce much pyruvate in order to replace traditional source is essential.

In Vietnam, there are several papers, which reported in pyruvate production by *Halomonas* strains. Ngo et al. (2018) reported nine strains isolated from mangrove forests of Cam Ranh, Khanh Hoa province that were able to synthesis and secreted pyruvate in the culture medium. However, the maximal pyruvate production only reached 0.11 g/L. According to Hoang et al. (2018), the pyruvate secretion can be achieved 21.02 g/L after 60 hours of cultivation by *Halomonas* sp. ND7.

The aim of this work was to isolate and determine biocharacteristics and pyruvate production of strain D34 from culture medium of *Dunalliella tertiolecta* for using in future industrialization of pyruvate.

## MATERIALS AND METHODS

### Source of bacteria

Samples were collected from culture medium of *D. tertiolecta*. This microalga which was deposited at Algal Biotechnology Department, Institute of Biotechnology, Vietnam Academy of Science and Technology, Vietnam. The composition of the growth medium of *D. tertiolecta* was Walne's medium with 12.5% NaCl (Walne, 1970).

### Baterial isolation and culture conditions

Strain D34 was isolated by the serial dilution technique using Glucose-Tryptone-Yeast (GTY) medium. This medium contained (per L): 1 g glucose, 0.5 g tryptone, 2 g yeast extract, 1 g $CaCl_2$, 100 g NaCl, 15 g agar as described by Tang et al. (2010) and incubated at 37°C for 1 week. Colonies were picked and repeatedly re-streaked onto GTY medium containing 5% NaCl. Strain D34 was maintained on GTY 5% NaCl slants at 4°C. Biomass for chemical and molecular studies was obtained by cultivation in 250 mL flasks with shaking (about 200 rpm) using liquid GTY 5% NaCl medium at 37°C for 3 days.

**Phenotypic characterization**

Gram staining was carried out using Gram kit (Nam Khoa Company, Vietnam). Colony morphology was observed on GTY medium containing 5% NaCl after incubation at 37°C for 6, 12, 24, 48 and 72 hours. Cell morphology and motility were examined by optical microscopy (Olympus CX21, Tokyo, Japan) and scanning microscopy- SEM (JSM-5410L, Jeol Company, Tokyo, Japan). For NaCl tolerance tests, strains were cultured at 37°C in GTY medium containing 0, 1, 3, 5, 10, 15, 20, 25 and 30% (w/v) NaCl. Growth was studied in GTY 5% NaCl medium at different temperatures (4, 10, 15, 20, 25, 30, 37, 40 and 45°C) and at pH 5–12 (at 1 pH unit intervals) using HCl or NaOH. Procedures followed for phenotypic characterization have been described previously by Mata et al. (2002) and Arahal et al. (2007).

Oxidase activity was determined by using Bactident ® Oxidase Kit (Merck, USA). Catalase activity was detected by the production of bubbles after the addition of a drop of 3% (v/v) $H_2O_2$. The exopolysaccharides were observed using the method of Azeredo & Oliveira (1996). Accumulation of polyhydroxybutyrate was determined by gas chromatography (Kawata et al., 2012).

The reduction of nitrate to nitrite, enzymatic activities, indol production, fermentation of D-glucose, its assimilation of D-mannose, D-mannitol, D-maltose, potassium gluconate, adipic acid and malic acid were analyzed using API 20NE microtest gallery systems (Biomerieux, Marcy l'Etoile, France).

**Chemotaxonomic characterization**

For analysis of fatty acids, strain D34 was cultivated at 37°C for 3 days on GTY medium containing 5% NaCl. Fatty acid analysis was performed by using a gas chromatograph (Hewlett-Packard 6890, New York, NY, USA) equipped with a flame-ionized detector and DB23 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) (Hong et al., 2011).

**Molecular analysis**

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene using primers 27F and 1525R (5′-AGAGTTTGATCMTGGCTCAG-3′; 5′-AAGGAGGTGATCCAGCC-3′) as the forward and reverse primers, respectively were performed. Sequence simility analysis was performed by comparing the 16S rRNA gene sequences of strain D34 from the GenBank database using the BLAST program (http:/www.ncbi.nlm.nih.gov/blast). Sequence data were aligned using the Clustal X software (Thompson et al., 1997). Phylogenetic trees were constructed using the neighbor-joining with the MEGA3 program (Kumar et al., 2004). The 16S rRNA gene sequences of strains *Oceanospirillum multiglobuliferum* (AB006764.1), *Zymobacter palme* (D1455.1) were used as the out-groups.

**Optimization of culture conditions**

For screening optimal medium, isolated strain D34 was cultured in flasks containing GTY 2% NaCl (pH 7.5), Marine Broth (Difco, Sparks Maryland, USA, pH 7.5), and SOT media (pH 9.4) which were supplemented 3% glucose. The SOT medium contained (g/L): $NaHCO_3$-12.6, $Na_2CO_3$-5.3, $K_2HPO_4$-0.5, $NaNO_3$-2.5, $K_2SO_4$-1.0, NaCl - 1.0, $MgSO_4$-0.2, $CaCl_2$-0.03, $FeSO_4 \cdot 7H_2O$-0.01, $Na_2EDTA \cdot 2H_2O$-0.09, and (mg/L): $H_3BO_3$ - 2.86, $MnSO_4 \cdot 7H_2O$-2.5, $ZnSO_4$-0.222, $CuSO_4 \cdot 5H_2O$-0.079, $Na_2MoO_4 \cdot 2H_2O$-0.021, $Co(NO_3)2 \cdot 6H_2O$-4.9 (Kawata et al., 2012). Growth and pyruvate production of strain D34 were analyzed after 3 days of cultivation at 37°C, with rotational shaking at a rate of 200 rpm.

The effect of glucose (10–25%), sodium nitrate (1–4%) concentrations in optimal culture medium, and culture temperature (25–

40°C) on cell growth and pyruvate production of strain D34 were determined using batch cultures in 250 mL Erlenmeyer flasks containing 30 mL culture medium with rotational shaking at a rate of 200 rpm.

**Analytical methods**

Cell growth was monitored by measuring the optical density at 600 nm ($OD_{600}$) using a spectrophotometer (Shimazu UV 1601 Spectrophotometer, Japan).

Pyruvate content was determined by Berntsson's method with several modifications (Berntsson, 1955). 1.5 mL samples of the culture were centrifuged at 12,000 rpm for 2 mins and collected supernatant. The resulting supernatants were used for determination of pyruvate. For pyruvate determination, 0.5 mL of 2% (v/v) solution of salicylaldehyde in 96% ethanol was added to test tube containing 1mL of supernatant and 1 mL of 25% NaOH. The mixture was mixed well and placed in water bath at 37°C. After 10 mins, the mixture was removed from the water bath. The absorbance at 470 nm was then determined. The blank and standards were prepared by adding sodium pyruvate solutions ranging in concentration from 0-0.5 M instead of supernatant samples.

All the experiments were conducted in triplicate (n=3 for each treatment).

## RESULTS AND DISCUSSION

### Morphological, physiological and biochemical characteristics of strain D34

Strain D34 was observed to be Gram-negative, aerobic, rod-shaped, approximately 0.45–0.60 μm wide and 1.25–5.10 μm long, occurring singly, non-motile, and flagellum-less (Fig. 1). Colonies were observed to be cream, circular, and smooth. This strain was able to produce exopolysaccharide, poly hydroxybutyrate, oxidase and catalase positive. The NaCl range for growth of strain D34 was 0.1–25% (w/v). Growth of the strain observed at 20–40°C and the pH ranged in 6–12.

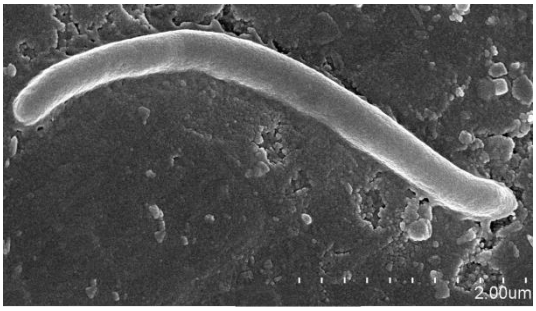


*Figure 1*. SEM images of strain D34. This strain was cultured in GTY 5% NaCl medium after 48 hours of cultivation under aerobic conditions (agitation speed: 200 rpm) at 37°C

Strain D34 was positive in nitrate reduction and negative in tests for indole production, arginine dihydrolase, β-galactosidase. Test for activity of urease, hydrolysis of gelatin were positive in D34 strain but ESCullin was negative. This strain utilized D-glucose, L-arabinose, D-manitol, malic acid, trisodium citrate as a carbon source, but did not utilize D-mannose, N-acetyl glucosamine, capric, apidic acid or phenylacetic acid. The main fatty acids of strain D34 were $C_{16:0}$ (35.59%), $C_{16:1\omega-7}$ (20.54%), $C_{18:1\omega-7}$ (30.14%), and $C_{12:0}$ (10.03% of total fatty acids). High levels of C16:0 and C18:1 in fatty acid profiles of these strains are correlated to the characteristic of the species in the genus *Halomonas* (Kim et al., 2010).

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain D34 belonged to the genus *Halomonas*. The phylogenetic tree showed that nearest phylogenetic neighbor of the isolate was *H. aquamarina*. The sequence similarity between strain D34 and *H. aquamarina* was 98.6%. Low 16S rRNA gene sequence similarity with respect to *H. aquamarina* as well as some characteristics showed that strain D34 was distinct from this type strain. Thus, based on results of the phylogenetic analysis,

morphological and chemotaxonomic investigations, we only supported the affiliation of the strain D34 to the genus *Halomonas* (Fig. 2).

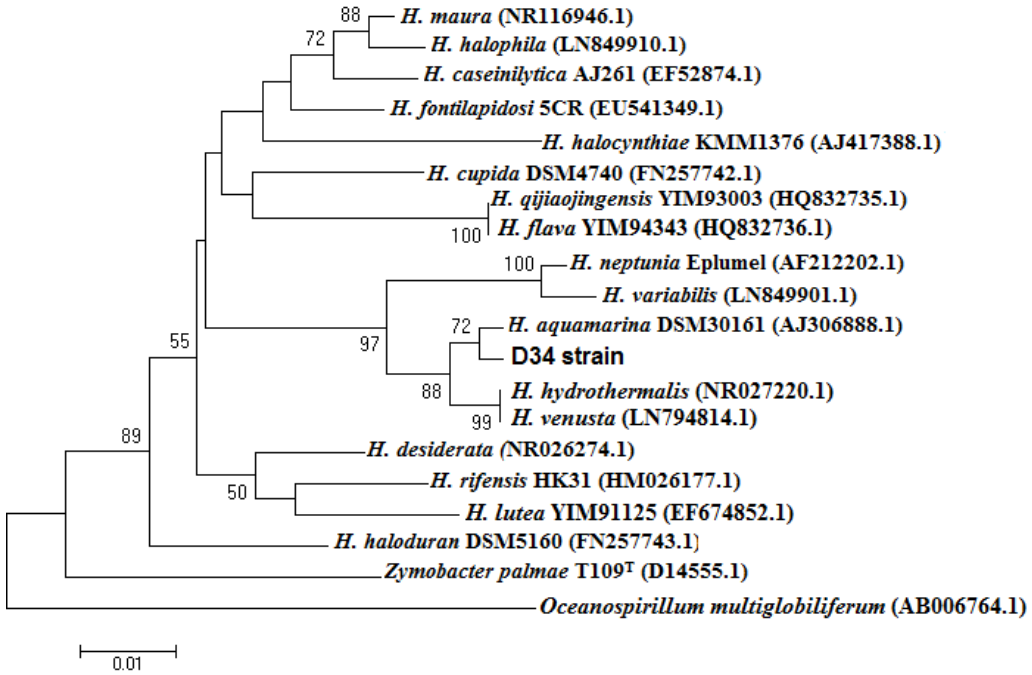


*Figure 2*. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain D34 and members of *Halomonas* genus. The numbers at the nodes indicate Bootrap values as percentages of 1,000 replicates. Values higher than 50% are expressed. Bar, 0.01 substations per 100 nucleotide position

**Growth and pyruvate production by strain D34 in different media**

Growth and pyruvate production of strain D34 were investigated in three media including GTY, MB and SOT medium plus 3% of glucose (Fig. 3). Marine Broth, used as an example of a commercially available high salt concentration medium. SOT medium is composed of only nutrient salts, while Marine Broth contains peptone and yeast extract in addition to nutrient salts. The highest level of $OD_{600\ nm}$ (6.23) and the highest pyruvate concentration (2.24 g/L) were observed when strain D34 was cultured in SOT medium plus 3% of glucose. These results suggested that SOT medium is suitable for pyruvate production by strain D34 and therefore, it was chosen for further study. When the strains applied for commercial pyruvate production, cheap artificial medium as modified SOT were preferred, thus further analysis were operated using modified SOT medium.

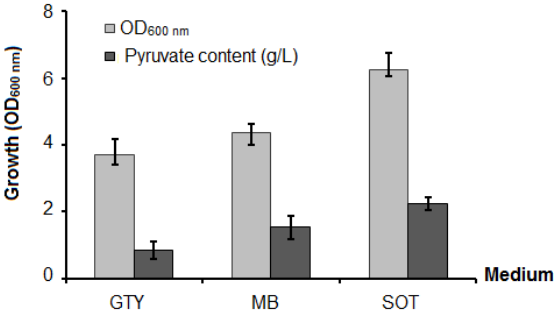


*Figure 3*. Growth and pyruvate production by strain D34 in different media

**Effect of glucose concentration on pyruvate production by strain D34**

The effect of glucose concentrations on the growth and pyruvate production of strain D34 were presented in Fig. 4.

The highest level of $OD_{600\ nm}$ (11.23) was observed after 60 h of cultivation when 10% glucose was added to SOT medium. However, addition of higher concentration of glucose (20–25%) inhibited the growth of strain D34 while the concentrations pyruvate production increased. The highest pyruvate concentration of 35.72 g/L was reached at 72 hours when 20% glucose was supplemented, which was 1.8–2.1 fold higher than that of medium adding 15% and 10% glucose, respectively.

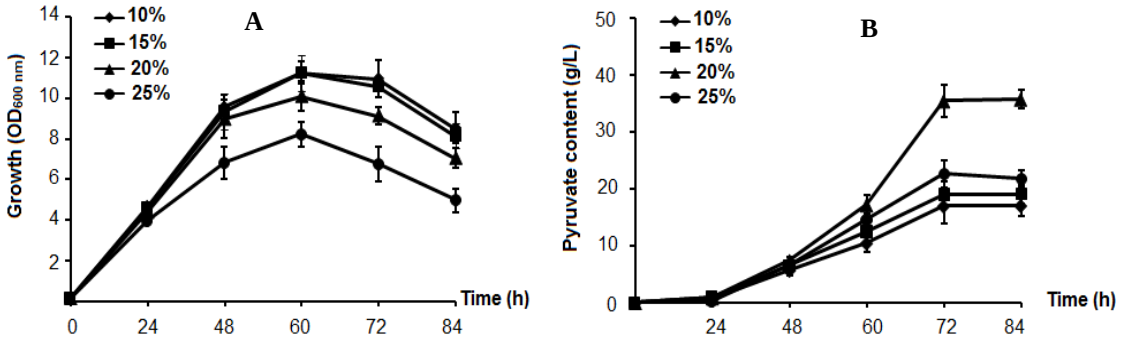


*Figure 4*. Effect of glucose concentration on growth (A) and pyruvate production (B) in *Halomonas* sp. D34

**Effect of nitrate concentration on pyruvate production by strain D34**

To increase in pyruvate production, we examined the effects of increasing sodium nitrate concentrations (10.0, 20.0, 30.0, and 40.0 g/L) in simple batch cultivations of strain D34 under aerobic conditions. The growth and pyruvate production by strain D34 are shown in Fig. 5.

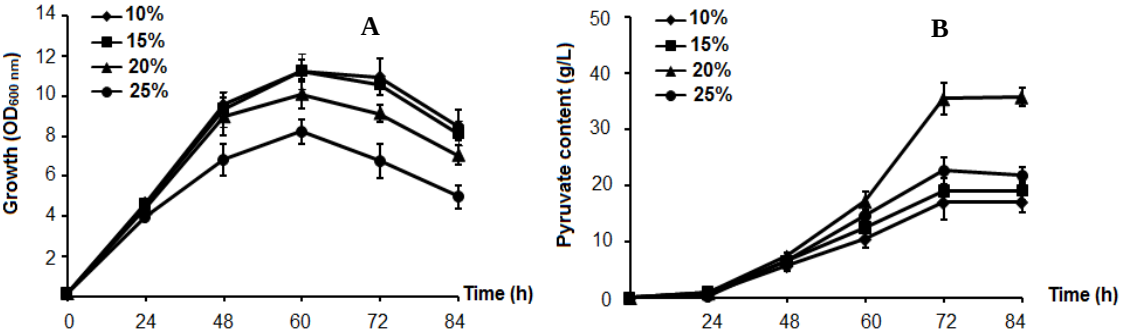


*Figure 5*. Effect of sodium nitrate concentration on growth (A) and pyruvate production (B) in *Halomonas* sp. D34

Maximal growth was observed after 60 hours of aerobic cultivation in all samples, whereas maximal pyruvate production was observed at 84 hours. The cell growth was decreased by increasing the concentration of sodium nitrate to 30 g/L. However, the

highest pyruvate production of 37.24 g/L was also obtained when a medium containing 30 g/L of sodium nitrate was used. This result obtained in strain D34 is in agreement with Kawata et al. (2016) who reported increased in sodium nitrate concentration promoted pyruvate secretion by strain KM-1. Thus, we used 30.0 g/L sodium nitrate in further experiments.

**Effect of culture temperature on pyruvate production by strain D34**

The effect of culture temperature on cell growth and pyruvate production of strain D34 was tested in the range of 28–45°C (Fig. 6). At 37°C, pyruvate production was the highest (36.41 g/L), whereas the highest level of $OD_{600\ nm}$ (18.71) was obtained at 32°C. Cultivation at 28, 32, and 40°C resulted in lower production of pyruvate than that at 37°C. Temperature is viewed as a major factor for cell growth and pyruvate production by *Halomonas*. Our results indicated that the cultivation temperature optimum for pyruvate secretion by strain D34 was 37°C. In comparison with *Halomonas* sp. KM-1, the optimal temperature for this strain is higher (at 40°C) than that of strain D34 (Kawata et al., 2016).

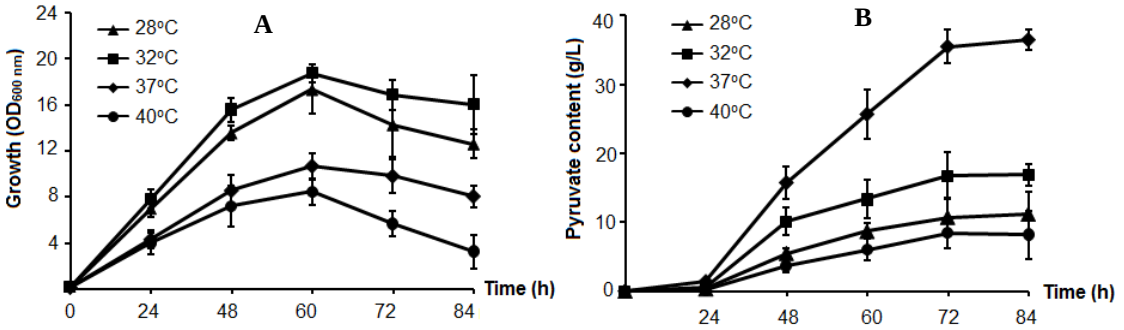


*Figure 6*. Effect of temparature on growth (A) and pyruvate production (B) in *Halomonas* sp. D34

**CONCLUSION**

In this study, halophilic pyruvate producing bacterial strain was isolated from the culture medium of *D. tertiolecta*. Maximal pyruvate secretion by strain was 37.24 g/L at 37°C with 20% glucose and 3% sodium nitrate after 84 hours of batch cultivation. The combination of phenotypic, chemotaxonomic characteristics and phylogenetic data indicated that this strain belonged to genus *Halomonas* and as designed D34. These data provided evidences for production of puruvate using novel wild-type strains.

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