Development of a system of high ornithine and citrulline production by a plant-derived lactic acid bacterium, *Weissella confusa* K-28

（植物由来乳酸菌 *Weissella confusa* K-28 における
オルニチン及びシトルリン高生産システムの構築）

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ABBREVIATIONS

ADI	arginine deiminase
AUC	area under curve
BLAST	basic local alignment search tool
CK	carbamate kinase
dNTP	deoxyribonucleotide triphosphate
DDBJ	DNA Data Bank of Japan
EDTA	ethylenediamine tetra-acetic acid
EtOH	ethanol
EPS	exopolysaccharide
GABA	gamma-aminobutyric acid
GRAS	generally recognized as safe
HPLC	high performance liquid chromatography
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
OD	optical density
OTC	ornithine carbamoyltransferase
PITC	phenyl isothiocyanate
TAE	tris-acetate-ethylenediamine tetra-acetic acid
TE	tris-ethylenediamine tetra-acetic acid
TEA	triethylamine
Tris	tris(hydroxymethyl)aminomethane
rDNA	ribosomal deoxyribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction

CONTENTS

INTRODUCTION

Lactic acid bacteria (LAB) are an order of gram-positive, acid-tolerant, generally nonsporulating, non-respiring, either rod-shaped or spherical microorganisms. Since LAB lack the ability of synthesizing porphyrins (heme, and components of respiratory chains) (Pessione *et al*, 2010), the bacteria can not generate ATP without external heme supplementation (Pessione *et al*, 2010). The LAB strains can only obtain ATP by fermentation, usually they generate the ATP from some sugars. Because LAB do not use oxygen for energy production, the bacteria easily grow under anaerobic conditions, but they can also grow in presence of oxygen. LABs are protected from oxygen-derived byproducts (*e.g.* peroxide radicals) because they have peroxidases and can detoxify the peroxidase radicals, although they lack the catalase enzyme which works on the H_2O_2 to convert H_2O and oxygen (Clarke and Cowan, 1952).

The LAB strains can be classified in various ways such as based on morphology, glucose utilization capacity, ability of growing at different temperatures, configuration of the production of lactic acid, growing ability at high salt concentrations, and acid or alkaline tolerance. Based on the optimum glucose utilization ability and fermentation capacity, LAB can be classified into two groups as follows: homo- and hetero-fermentative.

The homo-fermentative LAB species such as genus *Pediococcus*, *Lactococcus*, *Streptococcus*, and some *Lactobacillus* have the ability of producing lactic acid as the major end-product of glucose fermentation. The homo-fermentative LAB strains utilize the Embden-Meyerhof-Parnas pathway to produce two moles of lactate per mole of glucose and derive approximately twice as much energy per mole of glucose as hetero-fermentative lactic acid bacteria. On the other hand, hetero-fermentative LAB strains, such as the genus *Weissella*, *Leuconostoc*, and some *Lactobacillus*, can produce equimolar amounts of lactic acid, CO_2, and ethanol or acetate through the pentose or hexose monophosphate pathway. Various growth conditions may significantly alter the end-product formation by some LAB. These changes can be attributed to an altered pyruvate metabolism and/or the use of external electron acceptors such as oxygen or organic compounds.

The identification of LAB strain in past years were not time saving, accurate, and consistent process. However, to identify LAB strain, the typical phenotypic methods are most commonly used (Corsetti *et. al*. 2001) and reliable methods. Interestingly, due to the blessing of modern science in recent years, genetic techniques such as 16S rDNA sequencing have been established, and the method have allowed more consistent and accurate identification of individual strains (Buddhiman *et al*. 2008). Determining the

short sequences of 16S rDNA of LAB is considered as an easy way for the species determination of LABs isolates (Schleifer & Ludwig, 1995).

Healthcare effects of LAB strains on human

The human gastrointestinal tract (GIT) is considered as the inhabitant of a large number of bacterial species that comprise the intestinal microbiota (normal flora), and it is quite impossible for us to survive without their presence. The LAB strains are one of the major bacteria of the intestinal microbiota that help us to maintain the normal flora balance (Enrica *et al*. 2012). The colonized bacteria of humans play a wide range of functions such as i) manufacturing of some essential vitamins (vitamin B and K), ii) synthesis of digestive enzymes (like lactase), iii) making competition for the colonization sites with the notorious pathogenic bacteria, iv) ability of producing some antibacterial and antifungal products that protect from harmful pathogens, v) ability of producing of exopolysaccharides (EPSs) that have been shown as anti-carcinogenic (Wang *et al.* 2014) and vi) stimulates the development and activity of immune system (Ongol *et al.* 2012).

Beneficial effects of plant-derived LAB over animal-derived

The LAB strains have been traditionally used to produce fermented foods such as yogurt and cheese. They are generally recognized as safe (GRAS) microorganisms. The LAB strains have been reported to play beneficial immunomodulation, anti-obesity, and anti-allergic inflammation (Noda *et al.* 2018) roles in human health (Jin *et al.* 2010, Zhao *et al.* 2012).

The LAB strains are roughly classified into two groups: animal- and plant-derived LAB. The animal-derived LAB strains are found in the oral cavity and intestinal tract and are used to produce yogurt and cheese. The plant-derived LABs are found from many plant sources especially from the flowers, fruits, vegetables and medicinal plants. However, because the plant-derived LAB strains are found at the surface of plant petals, stems, and leaves, they are more adaptable to harsh conditions especially the habitats are nutritionally poor and contain alkaloids, tannins and polyphenols. We have claimed that plant-derived LAB strains are superior to animal-derived ones with respect to some health-promoting properties, such as immunomodulatory activity, tolerance to gastric and bile juices, and reducing constipation (Higashikawa *et al.* 2010).

LAB as probiotics

Since, the LAB strains have been considered as the beneficial bacteria for human microbiota that provide a lot of beneficial effects by maintaining or improving intestinal microbial balance. According to the WHO definition "probiotics are live microorganisms which, when administered in adequate amount confer a health benefits to the hosts" (Sanders *et al.* 2008), thus probiotic LAB should have the following criteria:

1. The LAB strains should be considered as GRAS. They should be genetically stable and resistant to antibiotics and the resistance should not be transmissible or inducible in nature.

2. The LAB strains should be able to survive and colonize at the human GIT, namely they should be able to resist against bile and/or gastric acid. Moreover, they should be stable against oxygen and enzymes like lysozymes.

3. The LAB strains should be able to adhere with the GIT. This ability improves the bacterial persistence and multiplication in the intestine, and then pathogens are competitively excluded from the mucosal surface.

4. The LAB strains should have good technological properties for manufacturing and incorporating into food products, without losing vital functionality and creating any unpleasant flavors.

5. Considering the minimum suggested therapeutic dose/day 10^8-10^9 cells, it is expected that LAB strains should meet the criterion of a minimum of 10^6 CFU/mL at the expiry date.

6. The LAB strains are obviously safe in food application and during the clinical use, even if in immune-compromised patients.

Mechanism of actions of probiotics

Probiotics give health benefits on the human. The several action mechanisms of probiotic bacteria, that improves the mucosal defense of the gastrointestinal tract, are as follows:

1. <u>Antimicrobial effect</u>:

Probiotics interfere the colonization of notorious pathogenic bacteria by reducing the intestinal luminal pH, by blocking the bacterial invasion and adhesion to the epithelial cell, and by producing the antimicrobial compounds such as bacteriocins and defensin,

organic acids, and hydrogen peroxide, The interaction between the LAB-mucosal epithelial cells and LAB-gut lymphoid cell enhances the gut immune response against ingested pathogens (Bourlioux *et al*. 2003; Mazahreh and Ershidat 2009).

2. Enhancement of mucosal barrier function against ingested pathogens:

Through the inflection of cytoskeletal and tight junctional protein phosphorylation, the production of mucin is increased. For epithelial binding sites, probiotic bacteria compete with the pathogenic bacteria and inhibit the colonization of strain, like *Salmonella* and *E. coli* directly or indirectly (through production of bioactive metabolites) (O′Shea *et al.* 2012). Based on these mechanisms, the probiotics may make benefits to the host in various diseases, for example, Type 1 diabetes (Watts *et al.* 2005).

3. Immunomodulation:

Particular strains of probiotics may influence both the innate and acquired immunity system with many kinds of cell types like epithelial cell, dendritic cell, and monocytes/macrophages and different types of lymphocytes (Natural killer cell, T-cell and T-cell redistribution) directly or secondarily (Walker, 2008), and can play vital role in human diseases. Such kind of action of probiotics are important for the elimination of neoplastic host cells (Soccol *et al.* 2010). Besides, the effects of probiotics on B-

lymphocytes and antibody production become an outcome of an increase in the IgA secretion and enhancing of response to vaccination (O'Flaherty *et al.* 2010). In recent years, it has been also reported that due to an increase of IgA in the bronchial mucosa, probiotics have the possibility of positive effect on the respiratory system by preventing and reducing the severity of respiratory infections (Wang *et al.* 2016).

L-Ornithine

L-Ornithine is a non-protein and free amino acid with a variety of significant functions and is used as a healthcare supplement. Because the ornithine is important for smooth functioning of the human immune system and liver, it has been used in the form of ornithine alpha-ketoglutarate (OKG) for treating liver disorder. Ornithine is a metabolic intermediate that plays a role in the urea cycle by detoxifying the ammonia to reduce the level of ammonia in the blood.

The oral administration of ornithine at regular interval has been reported to reduce stress and helps to improve the sleep quality of healthy workers (Miyake *et al.* 2014). The amino acid is also effective to reduce physical fatigue and increase mental capability *via* metabolic action (Konishi *et al.* 2015). Because ornithine also has the ability to promote

the synthesis and production of collagen, skin aesthetic and wrinkle improvement with ornithine has satisfied demands in the cosmetic industry. Furthermore, the amino acid has been regarded as an anti-obesity medicinal agent because the amino acid promotes the synthesis of body muscle through the promotion of growth hormone secretion.

Since, L-ornithine has been found to have multiple actions such as improved hepatic functions (Stewart and Walser, 1980) and increased growth hormone synthesis in the brain (Tujioka *et al.*, 2012). It is suggested that L-ornithine might be effective for maintaining the central and peripheral organs homeostatic functions.

For body weight control, the relative balance between food consumption and energy metabolism is very important. Anti-obesity agents are classified into the feeding inhibitors and metabolic activators or both depending on the mechanism of action (Konishi *et al.* 2015). Long-term ingestion of L-ornithine significantly is known to reduce the body weight, abdominal fat and food intake in rats.

Brackish-water bivalves (mainly *corbicula*) are the ingredient of well-known miso soup in Japan. They are considered as good for the liver if used for a long time. Experimental data have suggested that lipid and water-soluble fractions separated from the extract of *corbicula*, reduced the fatty liver and serum lipid concentrations in rats (Takeuchi *et al.* 1981). The extract of *Corbicula japonica* (シジミ) contained 20 mg

ornithine/100 g and dried *Lantinula edodes* (シイタケ) contained 10–170 mg ornithine/100 g and are known as vital natural sources of ornithine, although they do not contain sufficient amount of ornithine to satisfy the daily demand (400–1,000 mg). Additionally, since ornithine is a free amino acid and sufficient quantity of ornithine is not available in fish and meat, it is recommended to administer the ornithine or its supplement *via* oral administration.

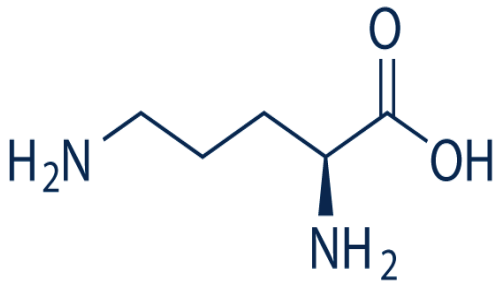


Fig. 1. Chemical structure of L-ornithine.

L-arginine

L-arginine, which is an essential amino acid, is important for medical and industrial applications. Arginine stimulates the synthesis of growth hormones, prolactin and insulin (Hwang & Lee, 2018). It helps to gain and maintain the muscle mass. The amino acid is important for human urea cycle and in ADI pathway in bacteria (Morris *et al.* 2006; Hwang & Lee, 2018).

ADI pathway and arginine metabolism

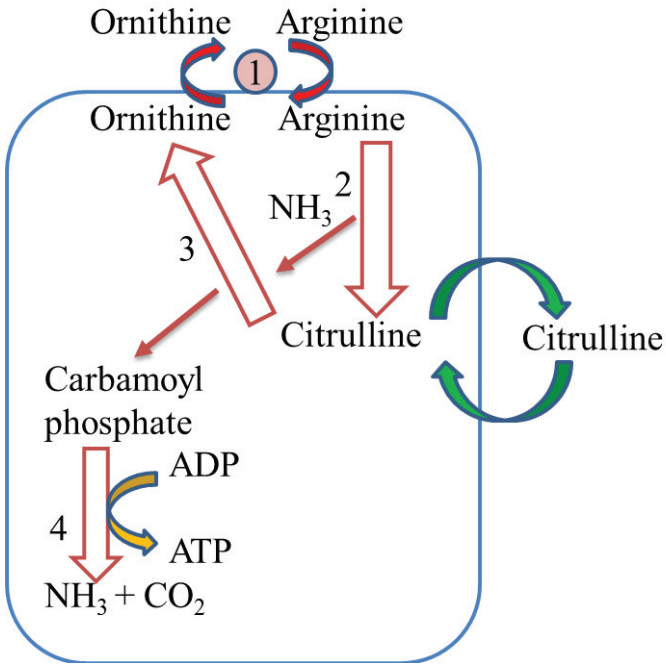
L-arginine could be degraded through the arginine deiminase (ADI) pathway by converting arginine to ornithine, CO_2, and NH_3 with the concomitant production of ATP, and the ADI pathway is widely found in Streptococcaceae (abdelal, 1979; Cunin *et al.* 1986). The ADI pathway in *Streptococcus* species is generally known as the arginine deiminase system (ADS) (Curran *et al*. 1988). The ADI pathway involves the enzymatic reactions as follows:

$$\text{Arginine} + H_2O \xrightarrow{\text{Arginine deiminase}} \text{Citrulline} + NH_3$$

$$\text{Citrulline} + P_i \xrightarrow{\text{Ornithine transcarbamylase}} \text{Ornithine} + \text{Carbamoyl-}\ P_i$$

$$\text{Carbamoyl-}\ P_i + ADP \xrightarrow{\text{Carbamate kinase}} NH_3 + CO_2 + ATP$$

In the ADI pathway, arginine is considered as the possible source of energy that can be tied to cellular growth, due to the formation of ATP. Casiano-Colon´ and Marquis suggested that a function of the ADI system is in the acid-base physiology of the bacteria (Casiano-Colon´ and Marquis, 1988).



1. Arginine/ornithine antiporter 2. Arginine deiminase
3. Ornithine transcarbamylase 4. Carbamate kinase

Fig. 2. Arginine deiminase pathway in lactic acid bacteria

The equimolar amounts of arginine are imported into the cells through ArcD antiporter in exchange for ornithine that is the waste product of arginine catabolism (ADI pathway). In the cytoplasm, arginine is degraded into citrulline and ammonia, with the help of arginine deiminase enzyme (ADI). The ultimate conversion of citrulline to ornithine and carbamoyl phosphate is performed with the help of catabolic ornithine transcarbamylase (OTC) enzyme. Finally, citrulline degrades into carbon dioxide and ammonium by carbamate kinase (CK) enzyme with the concomitant production of ATP.

Role of ornithine in urea cycle

Ornithine is the product of "urea cycle" along with urea and precursor of citrulline. That's why sometime the cycle is known as "ornithine cycle" (Barnes and Naylor, 1959). Two molecules of NH_3 and one CO_2 molecule are incorporated into this cycle. Various steps are involved in the cycle (Natesan *et al.* 2016) as follows:

Step 1: Ornithine combines with ammonium ion and carbon dioxide and was converted to carbamoyl phosphate.

$$NH_4^+ + CO_2 \xrightarrow{\text{carbamoyl phosphate synthase}} \text{carbamoyl phosphate}$$

Step 2: In the second step, ornithine combines with carbamoyl phosphate and was converted to citrulline and water.

$$\text{ornithine} + \text{carbamoyl phosphate} \xrightarrow{\text{ornithine carbamoyltransferase}} \text{citrulline} + H_2O$$

Step 3: In the third step, citrulline combines with second ammonia to form argininosuccinate.

$$\text{citrulline} + NH_3 \xrightarrow{\text{argininosuccinate synthase}} \text{argininosuccinate}$$

Step 4: In the fourth step, the formed argininosuccinate decomposed by argininosuccinate lyase to form arginine and fumarate.

$$\text{argininosuccinate} \xrightarrow{\text{argininosuccinate lyase}} \text{arginine} + \text{fumarate}$$

Step 5: In the final step, formed arginine later decomposed by arginase enzyme which is converted to urea and ornithine. ornithine is set free to repeat, and urea, which is formed in the liver, is carried by the blood to the kidneys for excretion process.

$$\text{arginine} \xrightarrow{\text{arginase}} \text{urea} + \text{ornithine}$$

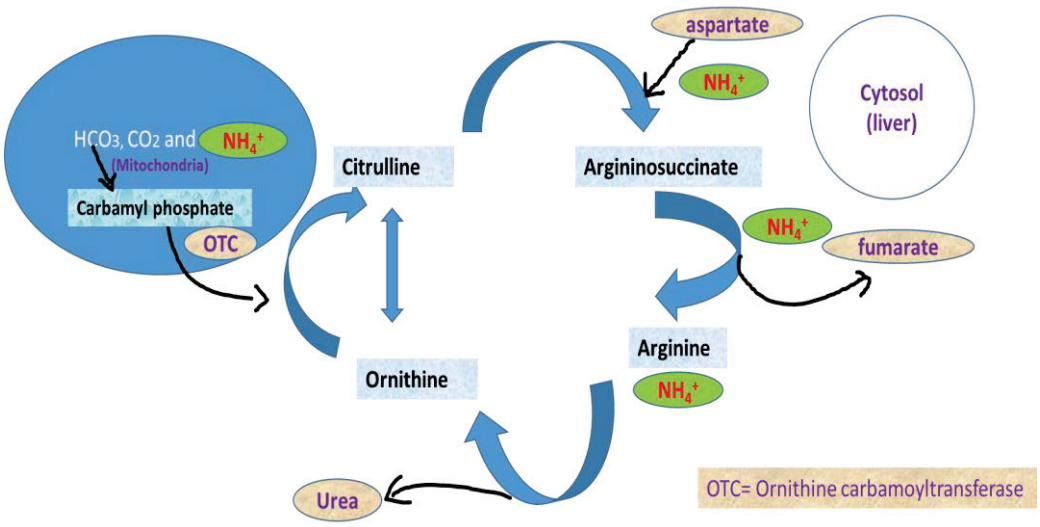


Fig. 3. Urea cycle in human body

The urea cycle, which is perform properly, is the key of removing the nitrogenous waste from the kidney. However, failure of the urea cycle to perform properly may cause the accumulation of ammonia level in the bloodstream resulting in serious liver disease, and in more severe cases, hepatic encephalopathy (synergistic effect of ammonia and other neurotoxins) which is characterized by severe brain injury may also be caused (Bleibel and Al-Osaimi, 2012).

L-Citrulline

L-citrulline is a non-protein amino acid, which has been first isolated and identified from the juice of watermelon (*Citrullus vulgaris*). Interestingly, citrulline is one of the most potent scavengers of the hydroxyl radical, and the watermelon accumulates citrulline simply, because this plant has no other way to allow the specific decomposition of the free hydroxyl radical (Moinard C & Cynober L, 2007). Although, in the early decades, citrulline had little interest to the scientists, but continuous research on it has created the value of citrulline because of its very specific metabolism. L-citrulline stimulates the muscle protein synthesis in a short-term low-protein diet.

Citrulline is a promising pharmaconutrient to provide nutritional support in malnourished patients, especially those who are aging and have sarcopenia. The amino acid is also the drug of choice in malnutrition patients especially the malnutrition associated with the intestinal failure. L-citrulline has been reported to retard high glucose–induced endothelial senescence in combination with arginine (Tsuboi *et al.* 2018). In sickle cell disease, citrulline has a vasodilation effect (Waugh *et al.* 2001). Moreover, orally administered citrulline improves the cardiac performance with the exercise (Waugh *et al.* 2001).

Senna obstusifolia

A short-lived (i.e. annual or biennial) shrub growing to up to 2.5 m tall, but usually less than 2 m in height. It is commonly known as Sicklepod.

Fig.4. *Senna Obstusifolia*

Distinctive Features

1. A short-lived sprawling shrub (annual or biennial)
2. Its compound leaves, which are borne on relatively short stalks and generally consist of two or three pairs of leaflets.
3. There is a small elongated projection which is located in between the lowest pair of leaflets on each leaf.
4. Its yellow flowers (10-15 mm across) are borne in pairs in the leaf forks which consist of five petals.
5. Its fruit is a slender in structure, sickle-shaped, pod (6-18 cm long and 2-6 mm wide) that is usually curved in the downwards.

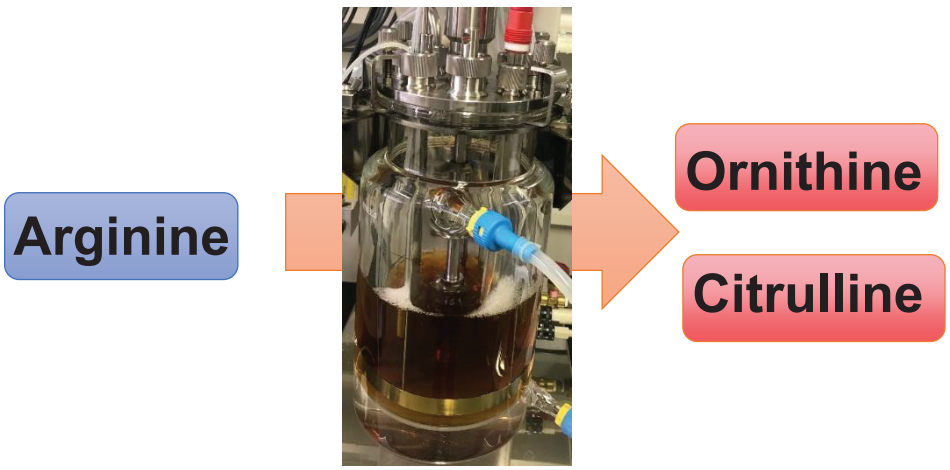
Flowering of the plant occurs mostly from late summer through to late winter (i.e. from March to August). The plant is traditionally used as a folk medicine by the local people.

More than 700 strains stored in the plant-derived LAB library established by us have been isolated from fruits, vegetables, flowers, and medicinal plants. We have found the LAB strains producing a large amount of γ-aminobutyric acid, bacteriocin, and EPS.

In the present study, the author isolated a plant-derived LAB strain, designated K-28, from the flower of *Senna obtusifolia* and identified it as *Weissella* (*W.*) *confusa*. The strain produces a large amount of ornithine when grown in the MRS medium supplemented with arginine. The author established the culture condition to produce a large amount of ornithine and citrulline in a fed-batch culture supplemented with arginine. To confirm whether the ornithine production is mediated by the arginine deiminase (ADI) pathway in the K-28 strain, we determined the ADI gene cluster using the primer walking method and evaluated the expression level.

OBJECTIVES

The objective of this study is to isolate the healthcare molecule-producing plant-derived LAB strains. During the study, the author successfully screened a high ornithine and citrulline producing strain, which is designated as K-28, identified as *Weissella confusa*. In the present study, the author confirmed that the K-28 strain produces ornithine and citrulline 18 ± 1 g/L and 10 ± 2 g/L, respectively, with a 100 ± 9% conversion rate when arginine was continuously fed into a jar fermenter. The amino acids production is mediated by the arginine deiminase (ADI) pathway, which is confirmed by determining the ADI gene cluster of K-28.



MATERIALS AND METHODS

Culture Conditions and Media

A medium, de Man, Rogosa, and Sharpe (MRS) medium (Becton, Dickinson and company, Franklin Lakes, NJ, U.S.A.), was used as a liquid culture medium to culture the LABs strain. A 1.5% (w/v) agar was added to the medium for replication and generation of colony.

Isolation of Ornithine-Producing LAB from Plant Sources

To obtain the desired LAB candidates, a plant sample (leaf, stalk, and flower), which was cut into small pieces, was suspended in the MRS medium and incubated anaerobically at the given temperature (28, 37, and 45°C) for 2–3 d. An aliquot of the cultured broth was spread on the MRS agar plate gently and then incubated at the given temperature. Each colony that appeared on the MRS agar plate was spread on a fresh MRS agar plate for purification. Gram-staining and catalase production tests were performed for the purified colony, followed by taxonomical identification. The ornithine and citrulline production by the isolated LAB strains was investigated by culturing for 72 h in the MRS medium supplemented with 0.5% (w/v) arginine.

Identification of LAB strains

1. Chromosomal DNA extraction

To extract chromosomal DNA from the LAB candidate, the bacterial cells were harvested from the 2 mL culture by centrifugation at 13,000 × *g* for 1 min at 4°C. The cell pellet was resuspended into a 500 μL of glucose-EDTA solution containing 40 mg/mL lysozyme (Wako Pure Chemicals Industries, Ltd.) together with 4 mg/mL achromopeptidase (Wako Pure Chemicals Industries, Ltd.), and then incubated for 2–3 h at 37°C in an incubator. After the resulting cell lysate was incubated for 3 h at 37°C, a 20 μL aliquot of 10% (w/v) SDS was added to the lysate. The proteins from the lysate were denatured by adding a 50 μL aliquot of 5 M $NaClO_4$ and removed by chloroform extraction. The chromosomal DNA was purified using an ethanol precipitation method. The resulting DNA was dissolved into 50 μL of TE buffer (10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) and stored at 4°C until use.

[Reagents]

Glucose-EDTA solution:

Glucose	: 50 mM
Tris-HCl (pH 8.0)	: 25 mM
EDTA . 2Na	: 10 mM
RNase	: 100 μg/mL

↓

Autoclave at 121°C, 20 min

2. Agarose gel electrophoresis

For gel electrophoresis, tris-acetate-EDTA (TAE) buffer containing 0.8% (w/v) agarose gel was prepared using 10 mg/mL of ethidium bromide. A total 10 μL DNA containing sample was prepared with a mixture of 5 μL sample, 4 μL distilled water, and 1 μL of 10 × dye. The resulted DNA bands were detected by ultraviolet light to check the chromosomal DNA purity.

[Reagents]

10 × dye:	Glycerol	40 (v/v) %
	Xylene cyanol	0.1 (w/v) %
	Bromophenol blue	0.1 (w/v) %
	Orange G	0.1 (w/v) %
	Distilled Water	q.s

TAE buffer:

A 50× concentrated TAE stock solution was made by weighing out 242 g of tris base and dissolved it in approximately 750 mL of deionized water. After adding 57.1 mL of glacial acid and 100 mL of 0.5 M EDTA (pH 8.0) to the diluted solution, the solution volume was adjusted to a final volume of 1 L. This stock solution was stored at room temperature.

The working solution of 1× TAE buffer is made by simply diluting the 50× stock solution in deionized water. This 1× solution contained 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.

λ/Hind III:

λ DNA	8 μg
10 × M buffer	20 μL
Hind III	24U
Distilled Water	final to 200 μL

↓ 37°C, 5 h

Water bath (55°C, 10 min)

↓

Added 20 μL of 10 × dye

↓

Stored at -20°C

3. 16S rDNA amplification

To amplify the 16S rDNA fragment, the reaction mixture was prepared, and the PCR reaction was performed as follows:

Compositions of the PCR mixture are following;

Sample	2.0 μL
10x blend Taq buffer	5.0 μL
dNTPs	5.0 μL
1525r primer (10 μM)	1.5 μL
27f primer (10 μM)	1.5 μL
Blend Taq	0.5 μl
Dimethyl sulfoxide	2.5 μL
Distilled water	32.0 μL
Total reaction volume	50.0 μL

[PCR primers for 16S rDNA amplification]

Forward primer: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′)

Reverse primer: 1525r (5′-AAGGAGGTGATCCAGCC-3′)

[PCR condition]

96°C (4 min) → 96°C (1 min), 50°C (20 sec), 72°C (1.5 min) × 30 cycles → 72°C (7 min) → 4°C (∞)

4. Gel electrophoresis for 16S rDNA fragments

To purify the 16S rDNA fragments, a 5 μL dye solution was added to the 50 μL PCR sample and then applied to the agarose gel. After the electrophoresis, the amplified DNA fragment in the gel was confirmed under UV light. The desired band was cut from the gel and purified as following section.

5. Purification of fragments

To purify 16S rDNA fragments, QIAquick (QIAGEN) gel extraction kit (Hilden, Germany) was used. According to the instruction manual, the 300 mg gel slice was taken into a micro-centrifuge tube, and 900 μL (3 vol.) buffer QG was added to the tube and incubated at 50°C for 10 min. After the gel slice put into the tube was dissolved in the buffer, a 300 μL (1 vol.) portion of isopropanol was added. The mixture was applied onto the QIAquick spin column and then the column was centrifuged 13,000 × *g* for 1 min at 4°C. The column was washed with 500 μL of buffer QG and washed twice with 750 μL of buffer PE. After drying the column by centrifugation, a 30 μL portion of the sterile water was added onto the center of the column membrane. The membrane-bound DNA fragments were eluted by centrifugation. For gel electrophoresis, 1 volume of loading dye solution with 5 volumes of the purified DNA sample were mixed and applied to the gel.

6. rDNA quantification

After purification, the concentration of purified DNA sample was measured by NANODROP 2000C (Thermo Scientific).

7. DNA Sequencing and Identification of LAB Strains

The nucleotide sequence was determined with the ABI PRISM 3130xl Genetic Analyzer using the BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The obtained sequence raw data were analyzed using ATGC software (GENETYX Corporation, Tokyo, Japan). The sequence data were compared with those of LAB species registered in the DNA Data Bank of Japan (DDBJ) database (https://www.ddbj.nig.ac.jp/index-e.html). Isolated LAB species were identified by using the BLAST algorithm-based homology search and the ClustalW program-based sequence alignment (http://clustalw.ddbj.nig.ac.jp/ index.php?lang=en).

Conditions for HPLC analysis of amino acids

The concentration of each amino acid was analyzed by using the phenyl isothiocyanate (PITC) derivatization method with a Wakopak Wakosil-PTC HPLC

column (4.6 × 250 mm; Wako Pure Chemicals Industries, Ltd., Osaka, Japan): a 10 μL aliquot of the culture supernatant was dried in *vacuo* in a microcentrifuge tube and then a 20 μL of pre-reaction reagent (ethanol / water / triethylamine (TEA) = 2 / 2 / 1) was added to the previous dried preparation. After drying a 20 μL portion of the reaction reagent (ethanol / water / TEA / PITC = 7 / 1 / 1 / 1) was added to the dried preparation and kept at room temperature and wait for 1 h to complete the reaction. After the reaction mixture was dried completely, the mixture was then dissolved into 1 mL of distilled water. The dissolved preparation was filtered with a 0.22 μm pore-size filter and finally stored at -20°C until use.

The analytical conditions were as follows: the mobile phase which was choose for amino acids analysis was a mixture of (A) PTC-Amino Acids Mobile Phase A (Wako Pure Chemicals Industries, Ltd.) and (B) PTC-Amino Acids Mobile Phase B (Wako Pure Chemicals Industries, Ltd.) with a linear gradient.

The programed gradient was instructed as follows: 0 to 20 min, 0–70% B. The column chromatography was performed with a flow rate of 1.0 mL/min at 40°C, and the eluates from the column were monitored to detect amino acids by using the UV detector at 254 nm wavelength.

Mutagenicity and Acute Oral Toxicity Tests

The mutagenicity test (umu-test) of the K-28 culture broth was performed by using a Umulac AT test kit (Protein Purify Ltd., Maebashi, Japan), in accordance with manufacturer's protocol. In the test kit, the induction of the *umu* gene was calculated (responsible for DNA damage) by the *umuC-lacZ* fusion gene expression in the *Salmonella enterica* serovar Typhimurium NM2009. If the sample enhanced the β-galactosidase activity more than twofold over the background, the test sample was considered to have the mutagen at the given concentration.

According to the OECD Guidelines for the Testing of Chemicals, Guidelines 420 (2001), an acute oral toxicity test on the K-28 cells was performed at Japan Food Research Laboratories. Slc:Wistar/ST male rats with age five weeks old were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were separated into two groups of five rats each and housed in polycarbonate cages room where temperature is controlled (20–26°C) with 12 h light–dark cycles. Rats were freely allowed to drinking water and a Labo MR Stock diet (Nosan Co., Yokohama, Japan). One research group was assigned to be a reference group and the other to a LAB-fed group, after one week of acclimation the fermented LAB cells were collected and resuspended in 15% (w/v) skim milk (2×10^{10} CFU/mL). The cell suspension (50 mL/kg) of the LAB cell was divided into three doses (20, 20, and

10 mL/kg) and orally administered by the oral route to the LAB-fed group of rats using a sterile stomach tube at 1 h intervals. In a control group, a 15% (w/v) skim milk without LAB cells was orally administered. The health status and behavior of the rats were recorded every day for 14 d, and after the animal experiment began, the body weight was measured at 1, 7, and 14 d. The rats were subjected to euthanize after the experimental period, and some extracted organs of the rats were histologically analyzed. By using Welch's t-test, the body weight difference between rats was analyzed. The same research was repeated using the female rats.

Culture Conditions for High Ornithine Production

1. Initial pH of the culture broth

To evaluate the initial pH effect on the ornithine productivity, the K-28 strain was cultured in the 0.5% (w/v) arginine-contained MRS medium with different initial pH ranges from 4.5 to 9.0 (0.5 interval) at 28°C. After the 3 days cultivation, ornithine productivity was measured.

2. Initial arginine concentration of the culture broth

To investigate the effect of initial arginine concentration to produce ornithine at a high level, the K-28 strain was cultured in the MRS medium at pH 6.5 and supplemented with various concentrations of arginine from 0.5-5.0% (w/v) (0.5 interval) at 28°C for 96 h. After the cultivation, ornithine productivity and arginine conversion ratio were measured.

3. Temperature and length of culture period

To determine the temperature for the vigorous growth, the K-28 strain was cultured in MRS medium (pH 6.5) containing 0.5% (w/v) arginine at the given temperature (20, 25, 28, and 37°C) for 120 h. An aliquot of the culture was collected at each 24 h interval for measuring the ornithine production until the 120 h. At the same time interval, the $OD_{600\ nm}$ of the culture broth was measured to monitor the bacterial cell growth.

Fed-Batch Cultivation Effect on High-yield Ornithine Production

For the small–scale cultivation test, the seed culture of the K-28 strain was inoculated [final 1% (v/v)] into the MRS medium adjusted at pH 6.5 and supplemented with 0.5% (w/v) arginine at 28°C without agitation. A 1 mL aliquot of culture broth was taken at each 12 h interval which was used for analysis of ornithine production. Until the 96 h

cultivation, the same volume of a 10% (w/v) arginine solution was added to the remaining culture broth after. The high-production of citrulline was observed until 96 h of cultivation, whereas the arginine conversion ratio to ornithine was reduced and the cell growth of K-28 strain was decreased after 96 h.

To test at a jar-fermenter scale cultivation, the seed culture of the K-28 strain was allowed to grown in 1 L of MRS medium (working volume of jar fermenter: 3 L) supplemented with 0.5% (w/v) arginine with continuous agitation at 28°C for 96 h. The pH of the culture medium was maintained at pH 6.5 by adding HCl or NaOH during the experimental period. After 12 h of cultivation, A 1 mL aliquot of cultured broth was taken after12 h cultivation and 10% (w/v) arginine [final concentration of arginine was 0.5% (w/v)] containing fresh MRS medium (pH 6.5) was added to the culture broth. Supplementation of the arginine solution and sampling of the culture broth were continued in each 12 h intervals for 96 h.

Sequencing Analysis of the ADI Gene Cluster

The ADI gene cluster of the K-28 strain was amplified by PCR using the ADI-F (5'-TAGAGAACCACTAAAGATC-3') and ADI-R (5'-CTTTTTTGCATCAGTTCCGA-

3') primers, which were designed based on conserved regions of the available ADI clusters from four *W. confusa* strains: AB3E41, DSM20196, LBAE C39-2, and MBF8-1 (RefSeq accession numbers NZ_FUWE01000018, NZ_JQAY01000001, NZ_CAGH01000001, and NZ_MNBZ01000002, respectively). The PCR was performed using a PrimeSTAR Max DNA Polymerase (Takara Bio, Inc., Shiga, Japan) under the following conditions: 35 cycles of 10 sec at 98°C, 5 sec at 60°C, and 30 sec at 72°C. The nucleotide sequence of the amplified fragment was determined by direct sequencing with ADI-F, ADI-R, and additionally designed inner primers. The open reading frames (ORFs) were predicted by using an ORF Finder program provided by NCBI at website (https://www.ncbi.nlm.nih.gov/orffinder/). The homology searches of the predicted ORFs were performed on the NCBI Web BLAST site (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST algorithm) utilizing the non-redundant database. The determined nucleotide sequence was deposited to DDBJ (accession number LC479518).

Extraction of Total RNA and Reverse Transcription (RT)-PCR Analysis

According to the manufacturer's protocol, total RNA extraction from *W. confusa* K-28 cell was performed using a NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) at each 12 h growth interval. In accordance with the

manufacturer's instructions, the complementary DNA (cDNA) was synthesized from total RNA using the ReverTra Aca qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan). In the RT-PCR analysis, using each primer set designed individually (Table 1) the target genes, *wkaA*, *wkaB*, *wkaC*, *wkaD*, and *wkaR*, were amplified where the synthesized cDNA was used as a template. A PrimeSTAR Max DNA Polymerase was used for the PCR reaction under the following conditions: 2 cycles of 5 sec at 98°C, 10 sec at 68°C, and 5 sec at 72°C; 2 cycles of 5 sec at 98°C, 10 sec at 66°C, and 5 sec at 72°C; 2 cycles of 5 sec at 98°C, 10 sec at 64°C, and 5 sec at 72°C; 2 cycles of 5 sec at 98°C, 10 sec at 62°C, and 5 sec at 72°C; and 27 cycles of 5 sec at 98°C, 10 sec at 60°C, and 5 sec at 72°C.

Table 1. List of designed primer sets used for RT-PCR analysis on ADI gene cluster of *W. confusa* K-28.

Target gene	Description	Forward primer (5'→3')	Reverse primer (5'→3')
wkaA	Arginine deiminase	CACTGATGAAAAGGTTCGTG	CGAACAATTCCTTAGCCAAC
wkaB	Ornithine carbamoyltransferase	TAGGACGTATGTTTGATGCC	GATCAATTCATCATCAGGCG
wkaC	Carbamate Kinase	GAAGAAGCAAAGACTGTTCG	GTAATTACCGTACCATCCCC
wkaD	Arginine ornithine antiporter	ACAACTTGTCTTTTCAAGCG	GATTGATGCGGTACACAATG
wkaR	Predicted transcriptional regulator	GTTGTTGTTAAAGGGGAGAC	AACTCACCGACTAGAATGTC
16S rRNA		TTGCTCAGATATGACGATGG	CCAATAAATCCGGATAACGC

Ornithine Production in Aerobic or Anaerobic Condition

To investigate the effect of aerobiosis and anaerobiosis for high-yield ornithine production, the K-28 strain was cultured in the MRS medium at pH 6.5 and supplemented with 0.5% (w/v) arginine at 28°C for 96 h. After the cultivation, ornithine production and the $OD_{600\ nm}$ was measured until the 96 h.

Total RNA extraction from *W. confusa* K-28 cell and complementary DNA (cDNA) was synthesized from total RNA according to the protocols described above. In the RT-PCR analysis, the target genes, *wkaA*, *wkaB*, *wkaC*, *wkaD*, and *wkaR*, were amplified using the synthesized cDNA as a template, with each primer set designed individually. The PCR reaction was performed using the above-mentioned conditions.

Ornithine Production without or with Arginine

To investigate whether the external arginine induce the ADI pathway, the K-28 strain was cultured in the MRS medium at pH 6.5 and supplemented with 0%, and 0.5% (w/v) arginine at 28°C for 48 h. In the RT-PCR analysis, the target genes, *wkaA*, *wkaB*, *wkaC*, *wkaD*, and *wkaR*, were amplified with the 16S-rRNA and compared each gene expression in 0% and 0.5% (w/v).

Ornithine Production and Arginine Conversion in different Juices Media

To investigate the ornithine production by K-28 strain was cultured in 0.5% (w/v) arginine-contained different 100% juices media (Pear, Pineapple, Mango, Carrot, Orange, Red grape, White grape and Apple) with initial pH 6.5 and cultured at 28°C. After the 60h cultivation, ornithine productivity and arginine conversion ratio to was measured.

RESULTS

Screening of an Ornithine-Producing LAB Strain

In the present study, the author isolated twenty LAB strains from several plant sources. A strain isolated from the flower of *Senna obtusifolia,* which was designated as K-28, produced a large amount of ornithine when cultured in the MRS medium supplemented with arginine. The entire 16S rDNA sequence of the K-28 strain was analyzed and compared with those of LABs registered in the DDBJ/EMBL/GenBank database. From the homology search, the K-28 strain was identified as *W. confusa*.

Evaluation of the Safety of K-28 Strain

Oral administration of the K-28 cells in mice was subjected to an acute toxicity test and shows that no significant changes in behavior or intake-related illnesses or deaths were observed. During the histological analysis, inflammatory symptoms and obvious differences did not appear in any organs of animals. Moreover, the *umu*-test demonstrates that the culture broth of the K-28 strain used for the experiment did not induce the mutagenicity.

Influence of Culture Conditions on High-yield Ornithine Production

In the present study, it was investigated the initial pH of the medium ranges 4.5–9.0 at 0.5 intervals as a culture condition to produce efficiently ornithine (Fig. 5), suggesting that the initial pH for high-yield production is suitable between pH 5.0 and 8.0. However, the production of ornithine was lower at pH 4.5 and pH 8.5–9.0.

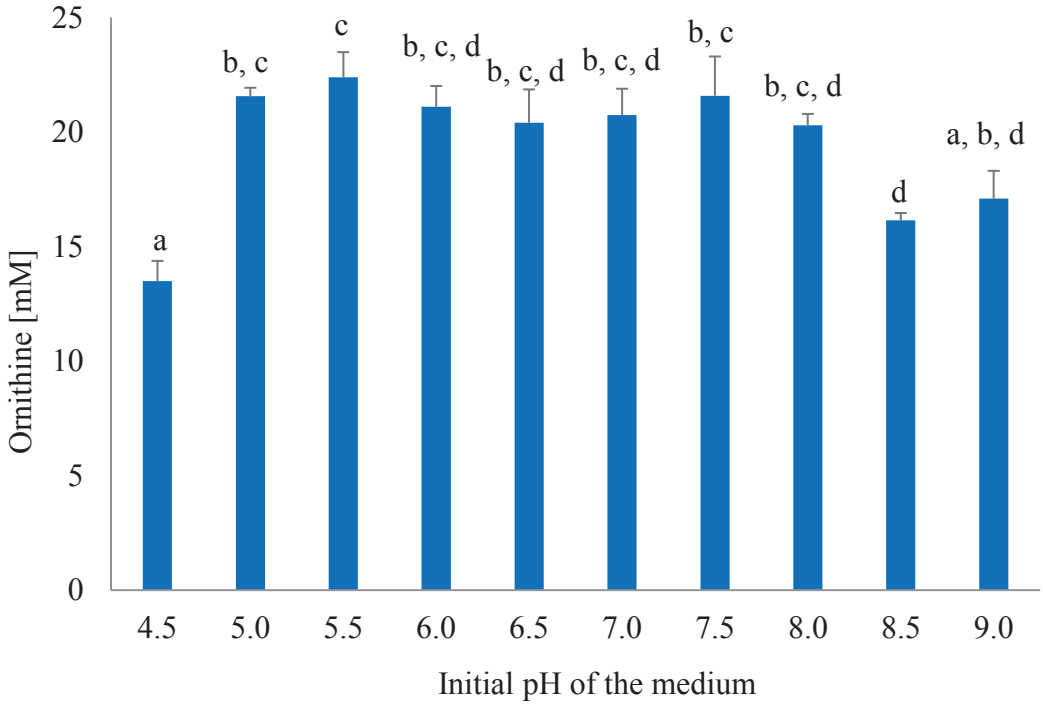


Fig. 5. Influence of initial pH of the culture medium supplemented with arginine 0.5% (w/v) on the ornithine production at 28°C for 72-h. The data were expressed as mean ± SE (n=3). The different characters mean statistically significant difference between groups (Tukey's HSD test, *p<0.05).

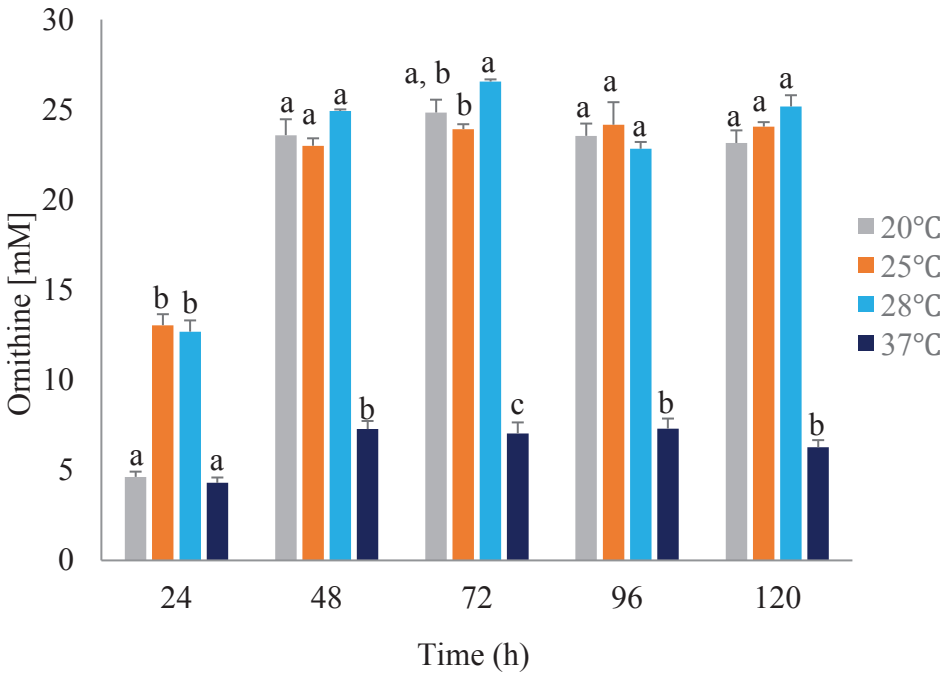


Fig. 6. influence of culture-temperature and -period on the ornithine production at initial pH 6.5 using the supplemented medium with 0.5% (w/v) arginine. The data were expressed as mean ± SE (n=3). The different characters mean statistically significant difference between temperatures (Tukey's HSD test, **p*<0.05) at each culture period.

It was also investigated the culture temperature and the cultivation period for the high ornithine production. Figure 6 suggests that, after 48 h or more cultivation, the K-28 strain produced the high level of ornithine at 20–28℃ but not at 37℃. Figure 7 shows that the production of ornithine and the cell growth may be considered as independent. In

addition, the cultivation at 28°C is suitable for the cell growth as well as ornithine production.

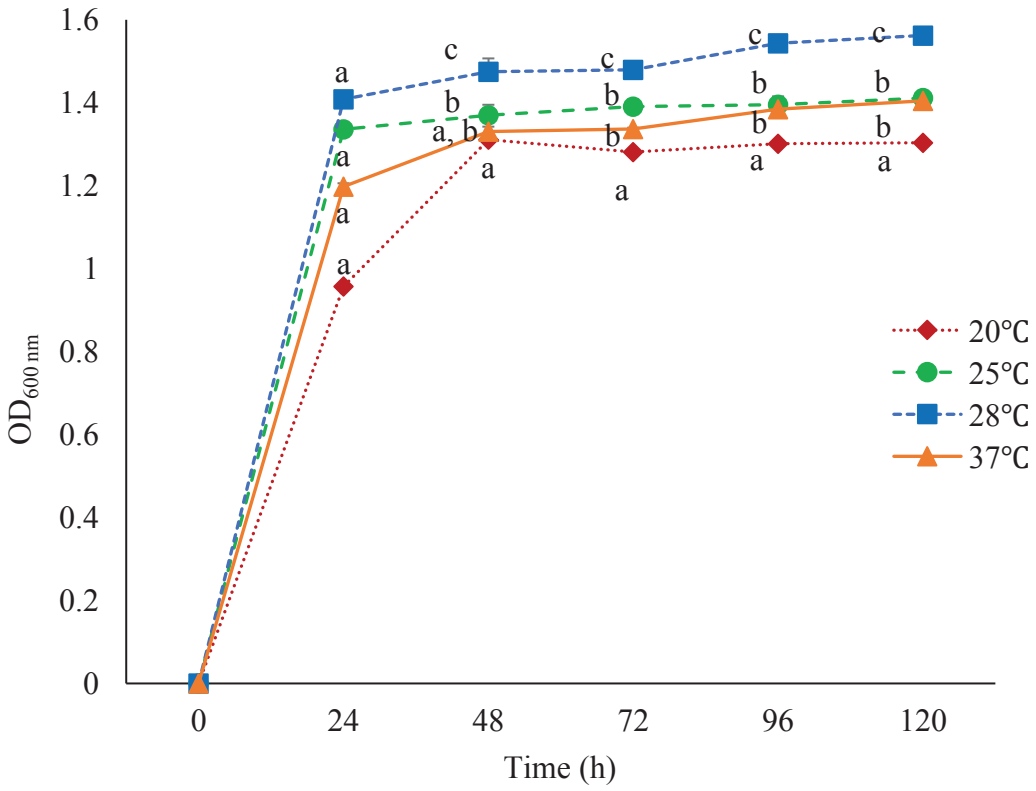


Fig. 7. Effect of culture-temperature and -period on the cell growth at 28°C using the medium at initial pH 6.5 and supplemented with 0.5% (w/v) arginine. The data were expressed as mean ± SE (n=3). The different characters mean statistically significant difference between temperatures (Tukey's HSD test, *p<0.05) at each culture period.

Ornithine is converted from precursor arginine *via* two enzymatic reactions: 1) arginine deiminase (EC 3.5.3.6) which converts arginine into intermediate citrulline and

ammonia, and 2) ornithine carbamoyltransferase (EC 2.1.3.3), which converts intermediate citrulline into ornithine and carbamoyl phosphate. Therefore, the initial concentration of the added arginine to the medium may affect the ornithine production. In this study, the effect of initial concentration of the supplemented arginine was examined within a range from 0.5 to 3.0% (w/v). Table 2 suggests that the production of ornithine is parallel with the initial arginine concentration added to the medium. However, the arginine conversion ratio to ornithine seems to be inversely related to the initial concentration of the supplemented arginine after the 48 h cultivation, suggesting that the high concentration of arginine added to the medium give rise to the saturation of conversion ratio from arginine to ornithine. In addition, a high-level of ornithine production was not significantly observed at more than the 96 h of cultivation (data not shown). Further, during the cultivation, period of 24–48 h at least about 80% of arginine was converted into ornithine.

Table 2. Effect of the initial arginine concentrations on the ornithine production (mM) and conversion ratio (%).

Cultivation time (h)		Initial Arg concentration (w/v %)					
		0.5	1.0	1.5	2.0	2.5	3.0
24	Orn (mM)	20.2 ± 0.5	40 ± 1	52 ± 4	81 ± 3	94 ± 3	123 ± 3
	Ratio (%)	(85 ± 3)	(84 ± 2)	(73 ± 5)	(86 ± 3)	(79 ± 2)	(87 ± 2)
48	Orn (mM)	22.9 ± 0.9	39 ± 1	54 ± 2	82 ± 5	103 ± 3	119 ± 3
	Ratio (%)	(92 ± 4)	(83 ± 4)	(76 ± 3)	(86 ± 5)	(87 ± 3)	(84 ± 2)
72	Orn (mM)	18.9 ± 0.7	36 ± 1	52.9 ± 0.2	79 ± 3	85 ± 3	115 ± 5
	Ratio (%)	(80 ± 3)	(76 ± 3)	(74.3 ± 0.3)	(83 ± 3)	(71 ± 3)	(81 ± 4)
96	Orn (mM)	23 ± 3	39 ± 3	51 ± 5	79 ± 8	94 ± 3	109 ± 2
	Ratio (%)	(100 ± 10)	(81 ± 5)	(71 ± 6)	(83 ± 8)	(80 ± 3)	(77 ± 1)

W. confusa K-28 was cultured in the MRS broth supplemented with arginine, with an initial pH of 6.5 at 28°C. The data were expressed as mean ± S.E. (n=3).

Fed-Batch Cultivation Effect

The author expected that the fed-batch culture supplemented with arginine result in the high ornithine production. Table 3 shows that the ornithine production was significantly increased, when the K-28 strain was cultured in the medium supplemented with arginine. In addition, after the ornithine production, a higher amount of citrulline was produced. Ornithine production seemed to be saturated at 48 h, whereas citrulline production would be started at 36–48 h. By keeping the arginine at a low concentration, the supplemented arginine was converted to intermediate citrulline in the bioconversion of arginine to ornithine.

It was evaluated the arginine-fed-batch effect on ornithine production using a 3 L jar fermenter by keeping the initial pH at 6.5. As shown in Table 4, during the cultivation period from 24 to 48 h, the arginine conversion ratio to ornithine drastically increased to over 100%. The additional arginine was immediately consumed to be converted to ornithine, whereas the citrulline production was enhanced in the long cultivation.

Table 3. Effect of fed-batch cultivation on ornithine and citrulline production by *W. confusa* K-28 in standing culture.

Cultivation time (h)	Amino acid concentrations in cultured broth (mM)			Total added Arg (mM / w/v %)	Amino acid conversion ratio (%)			OD_{600}
	Orn	Cit	Arg (unreacted)		Arg to Orn	Arg to Cit	Arg to (Orn + Cit)	
12	†13.6 ± 0.1[a]	†2.2 ± 0.6[a]	5.91 ± 0.06	23.7 / 0.5	58 ± 4	9 ± 3	67 ± 2	1.63± 0.02
24	71.6 ± 0.8[b]	5.1 ± 0.6[a]	1.1 ± 0.4	71.2 / 1.5	101 ± 1	7.2 ± 0.9	108 ± 2	2.17 ± 0.04
36	101 ± 9[b, c]	12 ± 1[b]	0.3 ± 0.1	118.7 / 2.5	85 ± 8	10.1 ± 0.9	95 ± 9	1.950 ± 0.008
48	120 ± 10[c, d]	31.2 ± 0.5[c]	0.19 ± 0.01	166.1 / 3.5	74 ± 6	18.7 ± 0.3	92 ± 6	1.58 ± 0.02
60	127 ± 8[c, d]	57 ± 2[d]	1.3 ± 0.1	213.6 / 4.5	60 ± 4	26.7 ± 0.8	86 ± 3	1.6 ± 0.1
72	139 ± 7[d]	73 ± 2[e]	0.5 ± 0.3	261.1 / 5.5	53 ± 3	27.9 ± 0.8	81 ± 4	1.741 ± 0.009
84	143 ± 7[d]	106.9 ± 0.5[f]	0.5 ± 0.2	308.6 / 6.5	46 ± 2	34.7 ± 0.2	81 ± 2	1.64 ± 0.02
96	128 ± 6[c, d]	125.8 ± 0.8[g]	0.34 ± 0.08	356.0 / 7.5	36 ± 2	35.4 ± 0.3	71 ± 2	-

W. confusa K-28 was cultured in the MRS broth supplemented with initial 0.5 (w/v) % arginine, with an initial pH of 6.5 at 28°C.

The 1.0% (w/v) arginine was added to the broth at each 12-h interval. The data were expressed as mean ± S.E. (n=3).

†Different characters mean statistically significant difference between cultivation length (Tukey's HSD test, $p<0.05$) at each amino acid.

Table 4. Effect of fed-batch cultivation on ornithine and citrulline production by *W. confusa* K-28 at jar-fermenter scale.

Cultivation time (h)	Amino acid concentrations in cultured broth (mM)			Total added Arg (mM / w/v %)	Amino acid conversion ratio (%)			CFU/mL (× 10^9)
	Orn	Cit	Arg (unreacted)		Arg to Orn	Arg to Cit	Arg to (Orn + Cit)	
12	†21 ± 7[a]	†3.4 ± 0.4[a]	10 ± 5	23.7 / 0.5	90 ± 30	14 ± 2	100 ± 30	8.2 ± 0.4
24	67 ± 4[b]	9 ± 2[a]	3 ± 2	47.5/ 1.0	142 ± 8	19 ± 5	160 ± 4	10 ± 2
36	80 ± 10[b]	14 ± 2[a, b]	0.9 ± 0.3	71.2 / 1.5	120 ± 20	19 ± 2	140 ± 20	9 ±1
48	96 ± 9[b, c]	21 ± 2[a, b, c]	1.2 ± 0.5	94.9 / 2.0	100 ± 10	22 ± 2	120 ± 10	8.3 ± 0.3
60	113 ± 7[b, c]	31 ± 3[b, c, d]	1.7 ± 0.8	118.7 / 2.5	96 ± 6	26 ± 3	122 ± 7	7.8 ± 0.6
72	115 ± 9[b, c]	39 ± 5[c, d, e]	2 ± 1	142.4 / 3.0	81 ± 6	27 ± 4	110 ± 10	7 ± 1
84	123 ± 7[c]	47 ± 6[d, e]	0.9 ± 0.2	166.1 / 3.5	74 ± 4	28 ± 3	103 ± 8	6 ±1
96	135 ± 8[c]	56 ± 9[e]	1.0 ± 0.2	189.9 / 4.0	71 ± 4	29 ± 5	100 ± 9	5.3 ± 0.9

W. confusa K-28 was cultured in the MRS broth supplemented with 0.5% (w/v) arginine, with an initial pH of 6.5 at 28°C. The 0.5 (w/v) % arginine was added to the broth at each 12-h interval. The data were expressed as mean ± S.E. (n=3).

† Different characters mean statistically significant difference between cultivation length (Tukey's HSD test, $p<0.05$) at each amino acid.

ADI Cluster Gene Organization and Analysis of Expression Patterns

To characterize the ADI (*wka*) gene cluster of the high-ornithine-producing *W. confusa* K-28, in the present study, the gene organization (Table 5) of the ADI cluster was analyzed.

Table 5. Gene organizaton of ADI gene cluster found in *W. confusa* K-28.

Gene	nt position	aa	Best Blast Homology (source)	Accession no.	Identity (%)
wkaA	194–1432	412	Arginine deiminase (*Weissella confusa* LBAE C39-2)	CCF30537	410/412 (99.5)
wkaB	1464–2510	348	Ornithine carbamoyltransferase (*Weissella confusa* LBAE C39-2)	CCF30536	348/348 (100)
wkaD	2513–3946	477	Arginine ornithine antiporter (*Weissella confusa* DSM 20196)	KRN24519	477/477 (100)
wkaC	3966–4907	313	Carbamate kinase (*Weissella confusa* 32)	RAU08366	313/313 (100)
wkaR	5017–5718	233	PucR family transcriptional regulator (*Weissella confusa* 32)	RAU08366	233/233 (100)

Organization of whole nucleotide sequence of ADI cluster by *W. confusa* K-28.

The K-28 strain which harbor an ADI cluster, consisting of five-genes with a 5.7 kb length, and the full-length nucleotide sequence with each gene direction is characterized where ATG is the start and TAA stop codon for each gene.

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TAGAGAACCACTAAAGATCCACAGCGATGTGGGTCTTTTTTTATACCTAAAAACGTTGGGTGTCATTATCTAAACAGATA          80
ATCTCTTGGTGATTTCTAGGTGTCGCTACACCCAGAAAAAAATATGGATAAAAGCAACCCACAGTAATAGATTTGTCTAT

AAAACCTTTGGCCATTGGGCCGTTTTGGAACGCTTTACTGAGTGACATAATAAAACTCGTAGCAAAGGAAGCGCTTACAT         160
TTTTGGAAACCGGTAACCCGGCAAAACCTTGCGAAATGACTCACTGTATTATTTTGAGCATCGTTTCCTTCGCGAATGTA
                                    wkaA⟶
                                      M  N  N  P  A  I  N  V  N  S  E  I  G  K  L  K
AAGCGTTTGTTAAAGTTCATAAGGAGGGGAACCATGAACAATCCTGCGATTAATGTTAACTCAGAGATTGGTAAGTTGAA         240
TTAGCAAACAATTTCAAGTATTCCTCCCCTTGGTACTTGTTAGGACGCTAATTACAATTGAGTCTCTAACCATTCAACTT

 S  V  L  L  K  R  P  G  A  E  V  E  N  I  T  P  D  T  M  E  R  L  L  F  D  D  I
GTCCGTGTTGCTTAAGCGACCTGGTGCTGAAGTTGAAAACATCACACCAGACACAATGGAACGTTTGTTGTTTGACGACA         320
CAGGCACAACGAATTCGCTGGACCACGACTTCAACTTTTGTAGTGTGGTCTGTGTTACCTTGCAAACAACAAACTGCTAT

   P  F  L  E  I  A  Q  Q  E  H  D  F  F  A  N  T  L  R  E  N  G  V  E  T  L  Y
TTCCATTCTTGGAGATCGCCCAACAAGAACACGACTTCTTTGCCAATACTTTGCGTGAGAACGGTGTTGAAACCCTTTAT         400
AAGGTAAGAACCTCTAGCGGGTTGTTCTTGTGCTGAAGAAACGGTTATGAAACGCACTCTTGCCACAACTTTGGGAAATA

I  D  D  L  A  V  E  A  L  D  T  D  E  K  V  R  E  A  F  V  Q  Q  Y  L  D  E  A
ATCGATGACCTAGCTGTTGAAGCTTTGGACACTGATGAAAAGGTTCGTGAAGCTTTCGTCCAACAATACTTGGATGAAGC         480
TAGCTACTGGATCGACAACTTCGAAACCTGTGACTACTTTTCCAAGCACTTCGAAAGCAGGTTGTTATGAACCTACTTCG

 G  Y  G  I  G  T  T  H  D  A  L  N  D  Y  L  K  T  F  N  T  R  D  L  V  T  K  L
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ACCAATGCCGTAGCCATGCTGAGTGCTACGTGACTTGCTAATGAACTTCTGCAAATTGTGCGCGCTGAACCAATGGTTCA

   Y  A  G  V  R  R  N  E  F  D  F  E  N  D  S  L  H  D  L  A  G  R  D  A  A  N
TGTATGCCGGGGTTCGTCGTAATGAATTTGATTTTGAAAATGATTCACTACACGATTTGGCTGGTCGTGATGCGGCAAAT         640
ACATACGGCCCCAAGCAGCATTACTTAAACTAAAACTTTTACTAAGTGATGTGCTAAACCGACCAGCACTACGCCGTTTA

P  F  L  L  D  P  L  P  N  A  Y  F  T  R  D  P  Q  A  S  I  G  N  G  M  T  I  N
CCATTCTTGTTGGATCCATTGCCAAACGCCTACTTCACACGTGACCCACAAGCATCAATTGGAAATGGTATGACCATTAA         720
GGTAAGAACAACCTAGGTAACGGTTTGCGGATGAAGTGTGCACTGGGTGTTCGTAGTTAACCTTTACCATACTGGTAATT

 H  M  T  F  K  A  R  Q  P  E  S  L  F  T  E  F  V  M  T  H  H  P  R  F  A  G  H
CCACATGACGTTTAAGGCTCGTCAACCAGAGTCATTGTTCACGGAATTCGTGATGACGCACCACCCACGCTTTGCTGGTC         800
GGTGTACTGCAAATTCCGAGCAGTTGGTCTCAGTAACAAGTGCCTTAAGCACTACTGCGTGGTGGGTGCGAAACGACCAG

   V  D  T  W  R  D  R  N  H  N  T  R  I  E  G  G  D  E  L  V  L  N  D  H  V  L
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TGCAACTATGGACCGCGCTGGCGTTGGTGTTGTGCGCATAACTTCCACCTCTGATTGAACACGAATTGCTGGTGCATGAA

A  I  G  V  S  Q  R  T  S  S  K  S  I  E  A  L  A  K  E  L  F  A  N  P  D  S  H
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CGATAACGACAAAGTGTTGCATGCAGAAGTTTCAGTTAGCTTCGCAACCGATTAATTAACAAGCGATTGGGTCTGTCAGT

 F  D  T  V  V  A  V  E  I  P  H  N  H  A  M  M  H  L  D  T  V  F  T  M  V  N  K
CTTTGATACTGTCGTTGCCGTTGAAATTCCACACAACCACGCGATGATGCACCTTGATACGGTCTTTACGATGGTTAATA        1040
GAAACTATGACAGCAACGGCAACTTTAAGGTGTGTTGGTGCGCTACTACGTGGAACTATGCCAGAAATGCTACCAATTAT

   Y  Q  F  T  V  F  P  G  I  M  D  E  G  G  K  M  N  I  F  I  M  T  P  G  P  D
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TCATAGTTAAATGCCAGAAGGGTCCTTAGTACCTACTCCCGCCATTCTACTTGTAGAAATAGTACTGCGGTCCGGGTCTA

G  T  V  K  L  A  H  R  T  D  L  G  A  T  L  K  E  V  L  G  L  S  E  L  D  L  I
GGCACGGTAAAGTTGGCTCACCGCACTGACCTTGGTGCAACGTTGAAGGAAGTGTTGGGACTTTCTGAGTTGGACTTGAT        1200
CCGTGCCATTTCAACCGAGTGGCGTGACTGGAACCACGTTGCAACTTCCTTCACAACCCTGAAAGACTCAACCTGAACTA

 E  T  G  S  G  D  A  I  V  A  P  R  E  Q  W  N  D  G  S  N  T  L  T  I  A  P  G
TGAGACTGGAAGCGGTGATGCGATTGTCGCACCTCGTGAGCAATGGAACGATGGTTCAAACACATTGACCATCGCACCAG        1280
ACTCTGACCTTCGCCACTACGCTAACAGCGTGGAGCACTCGTTACCTTGCTACCAAGTTTGTGTAACTGGTAGCGTGGTC

   E  V  V  T  Y  N  R  N  Y  V  S  N  E  L  L  R  R  H  G  I  L  V  H  E  V  I
GAGAAGTTGTGACCTACAACCGTAACTATGTAAGTAACGAATTGTTGCGTCGCCACGGCATTTTGGTTCACGAAGTCATT        1360
CTCTTCAACACTGGATGTTGGCATTGATACATTCATTGCTTAACAACGCAGCGGTGCCGTAAAACCAAGTGCTTCAGTAA
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S  S  E  L  S  R  G  R  G  G  P  R  C  M  S  Q  P  I  W  R  E  D  L  *
TCAAGTGAGTTGTCACGCGGTCGTGGTGGTCCACGTTGCATGTCTCAACCAATCTGGCGTGAAGATTTGTAAAACAGACA          1440
AGTTCACTCAACAGTGCGCCAGCACCACCAGGTGCAACGTACAGAGTTGGTTAGACCGCACTTCTAAACATTTTGTCTGT
                    wkaB ⟶
                       M  A  I  N  S  V  F  Q  G  R  S  L  L  A  E  K  D  F  T
AATAAAGGAGAAAAAGAAGAATCATGGCAATTAACTCAGTATTCCAAGGCCGTTCACTTTTGGCTGAAAAGGATTTTACC          1520
TTATTTCCTCTTTTTCAACTTCGTACCGTTAATTGAGTCATAAGGTTCCGGCAAGTGAAAACCGACTTTTCCTAAAATGG

S  A  E  I  Q  Y  L  V  D  F  G  L  H  L  K  E  L  Q  H  N  H  I  P  H  R  Y  L
TCAGCAGAAATTCAATATTTGGTTGATTTCGGTTTGCACTTGAAGGAGTTGCAACACAATCACATTCCACACCGTTACTT          1600
AGTCGTCTTTAAGTTATAAACCAACTAAAGCCAAACGTGAACTTCCTCAACGTTGTGTTAGTGTAAGGTGTGGCAATGAA

 E  G  K  N  I  A  L  L  F  E  K  T  S  T  R  T  R  S  A  F  T  T  A  A  I  E  L
GGAAGGTAAGAACATTGCTTTGTTGTTTGAAAAGACATCAACGCGTACCCGTTCAGCCTTCACCACGGCTGCTATCGAAC          1680
CCTTCCATTCTTGTAACGAAACAACAAACTTTTCTGTAGTTGCGCATGGGCAAGTCGGAAGTGGTGCCGACGATAGCTTG

  G  A  H  P  E  Y  L  G  S  N  D  I  Q  L  G  K  K  E  S  V  E  D  T  A  I  V
TAGGTGCTCACCCAGAATACCTTGGTTCAAACGACATCCAATTGGGTAAGAAGGAGTCTGTTGAAGACACTGCGATTGTC          1760
ATCCACGAGTGGGTCTTATGGAACCAAGTTTGCTGTAGGTTAACCCATTCTTCCTCAGACAACTTCTGTGACGCTAACAG

L  G  R  M  F  D  A  I  E  F  R  G  F  K  Q  S  D  V  E  G  L  A  K  Y  S  G  V
CTAGGACGTATGTTTGATGCCATTGAGTTCCGTGGCTTCAAGCAATCTGATGTTGAAGGATTGGCTAAGTACTCAGGTGT          1840
GATCCTGCATACAAACTACGGTAACTCAAGGCACCGAAGTTCGTTAGACTACAACTTCCTAACCGATTCATGAGTCCACA

 P  V  W  N  G  L  T  D  Q  W  H  P  T  Q  M  I  A  D  F  M  T  I  K  E  N  F  G
GCCAGTTTGGAACGGTTTGACTGACCAATGGCACCCAACACAAATGATCGCTGACTTTATGACAATCAAGGAAAACTTTG          1920
CGGTAAAACCTTGCCAAACTGACTGGTTACCGTGGGTTGTGTTTACTAGCGACTGAAATACTGTTAGTTCCTTTTGAAAC

  H  L  R  G  L  T  L  A  F  V  G  D  G  R  N  N  M  A  N  S  L  L  V  T  G  A
GCCACTTGCGCGGTTTGACGTTGGCCTTTGTTGGTGACGGTCGTAACAACATGGCGAACTCATTGTTGGTTACCGGTGCA          2000
CGGTGAACGCGCCAAACTGCAACCGGAAACAACCACTGCCAGCATTGTTGTACCGCTTGAGTAACAACCAATGGCCACGT

M  L  G  V  N  I  R  I  V  A  P  E  E  L  H  P  T  Q  E  V  V  D  L  A  N  G  F
ATGTTGGGTGTTAACATCCGCATTGTTGCACCTGAAGAGTTGCACCCAACGCAAGAAGTTGTTGACTTGGCAAATGGATT          2080
TACAACCCACAATTGTAGGCGTAACAACGTGGACTTCTCAACGTGGGTTGCGTTCTTCAACAACTGAACCGTTTACCTAA

 A  A  K  T  G  A  E  L  K  V  T  A  D  I  D  E  G  V  K  G  A  N  V  I  Y  T  D
TGCTGCTAAGACTGGTGCCGAGCTAAAGGTTACCGCTGATATCGACGAAGGTGTTAAGGGTGCTAACGTCATCTACACCG          2160
ACGACGATTCTGACCACGGCTCGATTTCCAATGGCGACTATAGCTGCTTCCACAATTCCCACGATTGCAGTAGATGTGGC

  V  W  V  S  M  G  E  S  D  W  E  T  R  V  K  L  L  T  P  Y  Q  V  N  M  D  M
ACGTTTGGGTATCAATGGGTGAGTCTGATTGGGAGACGCGTGTTAAGTTGTTGACGCCTTACCAAGTCAACATGGACATG          2240
TGCAAACCCATAGTTACCCACTCAGACTAACCCTCTGCGCACAATTCAACAACTGCGGAATGGTTCAGTTGTACCTGTAC

M  R  K  T  G  T  P  D  D  E  L  I  F  M  H  C  L  P  A  F  H  D  T  N  T  K  Y
ATGCGCAAGACTGGTACGCCTGATGATGAATTGATCTTCATGCACTGCTTGCCAGCCTTCCACGACACGAACACTAAGTA          2320
TACGCGTTCTGACCATGCGGACTACTACTTAACTAGAAGTACGTGACGAACGGTCGGAAGGTGCTGTGCTTGTGATTCAT

 G  E  D  V  K  E  Q  Y  G  I  T  E  M  E  V  T  D  E  V  F  R  S  K  Y  A  R  H
CGGTGAAGACGTCAAGGAACAATACGGTATCACTGAAATGGAAGTTACTGATGAAGTATTCCGTAGCAAGTACGCACGTC          2400
GCCACTTCTGCAGTTCCTTGTTATGCCATAGTGACTTTACCTTCAATGACTACTTCATAAGGCATCGTTCATGCGTGCAG

  W  D  E  A  E  N  R  K  H  S  I  K  A  I  M  A  A  T  L  G  N  L  F  I  P  D
ACTGGGACGAGGCTGAAAACCGCAAGCACTCAATCAAGGCCATCATGGCTGCAACGTTGGGTAACTTGTTCATTCCAGAT          2480
TGACCCTGCTCCGACTTTTGGCGTTCGTGTGTTAGTTCCGGTAGTACCGACGTTGCAACCCATTGAACAAGTAAGGTCTA
                                wkaD ⟶
V  P  A  L  E  E  A  G  K  *     M  A  D  T  K  D  K  G  I  A  L  P  A  L  V  A
GTACCTGCGCTTGAAGAAGCCGGTAAGTAATTATGGCAGATACTAAGGATAAAGGAATTGCTTTACCTGCCTTAGTAGCG          2560
CATGGACGCGAACTTCTTCGGCCATTCATTAATACCGTCTATGATTCCTATTTCCTTAACGAAATGGACGGAATCATCGC

L  V  V  S  S  A  I  G  A  G  I  F  D  L  P  A  T  L  A  Q  G  A  T  P  G  A  A
CTAGTCGTTAGTTCTGCAATCGGTGCTGGAATCTTTGATTTGCCAGCGACTTTGGCCCAGGGTGCAACACCTGGAGCGGC          2640
GATCAGCAATCAAGACGTTAGCCACGACCTTAGAAACTAAACGGTCGCTGAAACCGGGTCCCACGTTGTGGACCTCGCCG

 I  L  A  W  V  I  T  G  F  G  I  L  M  L  A  L  S  L  N  H  L  V  L  T  K  P  E
CATTCTTGCTTGGGTAATTACGGGATTTGGTATCTTGATGTTGGCATTGAGTCTTAATCATTTGGTGCTGACGAAGCCTG          2720
GTAAGAACGAACCCATTAATGCCCTAAACCATAGAACTACAACCGTAACTCAGAATTAGTAAACCACGACTGCTTCGGAC
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  L  T  G  V  S  D  Y  A  R  A  G  F  G  D  F  A  G  F  I  S  G  W  G  Y  W  L
AACTAACTGGTGTTTCTGACTATGCGCGTGCCGGGTTTGGTGATTTTGCTGGATTTATTTCTGGCTGGGGATACTGGTTG                2800
TTGATTGACCACAAAGACTGATACGCGCACGGCCCAAACCACTAAAACGACCTAAATAAAGACCGACCCCTATGACCAAC

S  A  W  L  G  N  V  A  F  A  T  M  L  M  S  A  L  G  Y  F  F  P  P  L  K  S  G
TCAGCTTGGCTAGGTAACGTTGCCTTCGCAACGATGCTGATGTCAGCGTTAGGTTACTTCTTTCCACCACTTAAATCTGG                2880
AGTCGAACCGATCCATTGCAACGGAAGCGTTGCTACGACTACAGTCGCAATCCAATGAAGAAAGGTGGTGAATTTAGACC

 N  S  V  S  A  I  I  V  A  S  I  V  S  W  A  L  T  L  L  V  T  H  G  I  E  S  A
AAACAGCGTCAGCGCCATTATTGTCGCCTCAATTGTGTCATGGGCACTAACGTTACTTGTCACACACGGTATTGAGTCGG                2960
TTTGTCGCAGTCGCGGTAATAACAGCGGAGTTAACACAGTACCCGTGATTGCAATGAACAGTGTGTGCCATAACTCAGCC

  A  A  I  N  T  I  V  T  V  T  K  L  V  P  I  F  A  F  V  V  I  A  F  L  S  M
CTGCAGCCATCAATACAATTGTGACGGTGACTAAGTTGGTACCGATCTTCGCCTTTGTTGTCATTGCTTTCTTAAGTATG                3040
GACGTCGGTAGTTATGTTAACACTGCCACTGATTCAACCATGGCTAGAAGCGGAAACAACAGTAACGAAAGAATTCATAC

K  A  G  V  F  T  A  H  F  W  Q  N  M  T  T  N  V  Q  G  Q  L  V  F  S  S  A  T
AAGGCGGGGGTCTTCACGGCACACTTCTGGCAAAACATGACCACTAATGTTCAAGGACAACTTGTCTTTTCAAGCGCAAC                3120
TTCCGCCCCCAGAAGTGCCGTGTGAAGACCGTTTTGTACTGGTGATTACAAGTTCCTGTTGAACAGAAAAGTTCGCGTTG

 G  A  G  I  F  A  Q  V  K  G  C  V  M  A  M  M  W  V  F  V  G  I  E  G  A  T  M
GGGTGCGGGTATCTTTGCGCAAGTGAAGGGTTGTGTTATGGCCATGATGTGGGTCTTCGTTGGAATTGAAGGCGCTACGA                3200
CCCACGCCCATAGAAACGCGTTCACTTCCCAACACAATACCGGTACTACACCCAGAAGCAACCTTAACTTCCGCGATGCT

  M  A  S  R  A  K  K  K  S  D  A  G  K  A  T  I  I  G  L  V  S  L  L  I  I  Y
TGATGGCGTCACGTGCCAAGAAGAAGTCCGATGCAGGAAAGGCCACGATTATCGGCTTGGTGTCATTGCTGATTATCTAC                3280
ACTACCGCAGTGCACGGTTCTTCTTCAGGCTACGTCCTTTCCGGTGCTAATAGCCGAACCACAGTAACGACTAATAGATG

M  V  V  S  I  L  P  Y  G  Y  L  S  L  E  Q  L  R  G  M  Q  H  P  A  L  L  Y  L
ATGGTTGTTTCGATTTTACCTTATGGTTATTTGTCACTAGAACAATTGCGTGGGATGCAACACCCAGCGCTACTGTACCT                3360
TACCAACAAAGCTAAAACGGAATACCAATAAACAGTGATCTTGTTAACGCACCCTACGTTGTGGGTCGCGATGACATGGA

 F  E  S  M  T  G  R  L  G  G  A  F  I  S  V  G  L  I  I  S  I  L  G  A  W  L  S
GTTTGAAAGCATGACAGGTCGTTTGGGTGGTGCGTTCATCAGTGTTGGCTTGATTATTTCAATTTTGGGAGCTTGGTTGT                3440
CAAACTTTCGTACTGTCCAGCAAACCCACCACGCAAGTAGTCACAACCGAACTAATAAAGTTAAAACCCTCGAACCAACA

  W  T  M  L  P  V  E  A  T  S  L  M  A  E  Q  R  L  L  P  K  W  F  G  T  L  N
CATGGACGATGTTGCCAGTTGAAGCAACATCTTTGATGGCAGAACAACGCTTGCTACCAAAGTGGTTCGGTACGTTAAAC                3520
GTACCTGCTACAACGGTCAACTTCATTGTAGAAACTACCGTCTTGTTGCGAACGATGGTTTCACCAAGCCATGCAATTTG

K  Y  R  A  P  Q  H  S  L  L  L  T  Q  I  L  I  Q  I  F  L  I  T  L  L  F  T  D
AAGTACCGCGCCCCACAACACTCATTGTTGTTGACGCAAATTTTGATTCAAATTTTCCTTATCACGTTGCTCTTTACTGA                3600
TTCATGGCGCGGGGTGTTGTGAGTAACAACAACTGCGTTTAAAACTAAGTTTAAAAGGAATAGTGCAACGAGAAATGACT

 Q  A  Y  N  F  A  Y  S  L  C  T  A  S  I  V  V  C  Y  V  L  V  G  A  Y  E  L  K
TCAGGCGTACAACTTTGCGTACTCATTGTGTACCGCATCAATCGTTGTCTGCTACGTATTGGTTGGAGCGTATGAACTGA                3680
AGTCCGCATGTTGAAACGCATGAGTAACACATGGCGTAGTTAGCAACAGACGATGCATAACCAACCTCGCATACTTGACT

  L  G  I  G  S  K  K  W  S  V  I  L  P  G  L  I  T  V  V  F  E  V  A  A  I  V
AGCTTGGTATCGGGTCAAAGAAGTGGTCCGTTATCTTGCCAGGTTTGATTACAGTTGTCTTTGAAGTCGCTGCGATTGTC                3760
TCGAACCATAGCCCAGTTTCTTCACCAGGCAATAGAACGGTCCAAACTAATGTCAACAGAAACTTCAGCGACGCTAACAG

F  S  G  L  Q  Y  L  W  L  C  T  I  L  Y  V  L  G  F  V  L  Y  Y  R  A  R  K  E
TTCTCAGGTTTGCAGTACTTGTGGTTGTGCACGATTCTTTATGTTTTGGGATTTGTGTTGTACTACCGAGCACGCAAGGA                3840
AAGAGTCCAAACGTCATGAACACCAACACGTGCTAAGAAATACAAAACCCTAAACACAACATGATGGCTCGTGCGTTCCT

 D  G  K  Q  V  T  K  S  E  Y  V  M  M  G  I  V  L  L  L  A  V  A  A  V  V  A  L
AGACGGCAAGCAAGTGACCAAGTCAGAGTACGTGATGATGGGAATTGTCTTGTTGCTCGCAGTAGCAGCAGTTGTTGCCT                3920
TCTGCCGTTCGTTCACTGGTTCAGTCTCATGCACTACTACCCTTAACAGAACAACGAGCGTCATCGTCGTCAACAACGGA
                                                    wkaC ⟶
  A  T  G  S  L  K  I  *                    M  T  K  R  I  V  V  A  L  G  G  N
TAGCCACGGGTAGCTTAAAGATTTAAGAGATTGGAGAAATGTAATATGACAAAGCGAATTGTTGTTGCACTTGGTGGAAA                4000
ATCGGTGCCCATCGAAGGGCTAAATTCTCTAACCTCTTTACATTATACTATTTCGCTTAACAACAACGTGAACCACCTTT

 A  I  L  T  D  D  P  S  A  E  A  Q  Q  T  A  L  Q  Q  T  A  K  Y  L  V  N  F  L
TGCGATTTTGACGGATGATCCAAGTGCAGAAGCCCAACAAACAGCCTTACAACAAACGGCCAAGTATTTGGTTAACTTTT                4080
ACGCTAAAACTGCCTACTAGGTTCACGTCTTCGGGTTGTTTGTCGGAATGTTGTTTGCCGGTTCATAAACCAATTGAAAA
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  E  M  G  D  V  Q  L  V  L  T  H  G  N  G  P  Q  V  G  N  L  V  L  Q  Q  L  D
TGGAAATGGGGGATGTCCAACTTGTTTTGACGCACGGAAACGGGCCCCAAGTTGGTAACCTCGTTTTGCAACAATTGGAT      4160
ACCTTTACCCCCTACAGGTTGAACAAAACTGCGTGCCTTTGCCCGGGGTTCAACCATTGGAGCAAAACGTTATTAACCTA

G  S  S  A  K  N  P  A  M  P  L  D  T  V  G  A  M  T  Q  G  E  I  G  L  W  L  A
GGCAGCTCAGCAAAGAACCCAGCCATGCCATTGGATACTGTTGGGGCGATGACCCAAGGTGAAATTGGCTTGTGGTTGGC      4240
CCGTCGAGTCGTTTCTTGGGTCGGTACGGTAACCTATGACAACCCCGCTAAGGGGTTCCACTTTAACCGAACACCAACCG

 D  A  L  N  E  E  I  I  S  R  G  L  D  Q  K  V  A  T  V  L  T  R  T  V  V  D  A
AGATGCTTTGAACGAAGAGATTATTAGTCGCGGGTTAGATCAAAAGGTCGCAACTGTTTTGACCCGTACAGTGGTCGATG      4320
TCTACGAAACTTGCTTCTCTAATAATCAGCGCCCAATCTAGTTTTCCAGCGTTGACGGGGCTGGGCATGTCACCAGCTAC

  N  D  A  A  F  E  Q  P  T  K  P  I  G  P  F  Y  T  A  E  E  A  K  T  V  R  D
CAAACGATGCCGCCTTTGAGCAACCAACTAAGCCAATTGGACCATTCTACACAGCCGAAGAAGCAAAGACTGTTCGTGAT      4400
GTTTGCTACGGCGGAAACTCGTTGGTTGATTCGGTTAACCTGGTAAGATGTGTCGGCTTCTTCGTTTCTGACAAGCACTA

E  H  P  D  W  T  I  V  E  D  A  G  R  G  Y  R  R  V  V  P  S  P  K  P  I  S  I
GAGCACCCAGATTGGACTATCGTTGAGGACGCTGGTCGCGGCTACCGTCGTGTTGTGCCATCTCCAAAGCCAATCAGTAT      4480
CTCGTGGGTCTAACCTGATAGCAACTCCTGCGACCAGCGCCGATGGCAGCACAACACGGTAGAGGTTTCGGTTAGTCATA

 M  E  A  D  A  I  R  T  L  A  Q  E  G  V  T  L  I  A  A  G  G  G  G  V  P  V  V
TATGGAGGCGGACGCGATTCGAACGCTTGCGCAAGAAGGGGTAACGCTAATTGCAGCCGGCGGTGGTGGTGTCCCAGTTG      4560
ATACCTCCGCCTGCGCTAAGATTGCGAACGCGTTCTTCCCCATTGCGATTAACGTCGGCCGCCACCACCACAGGGTCAAC

  R  K  D  N  D  I  T  G  V  E  A  V  I  D  K  D  F  T  A  A  K  L  A  E  L  V
TTCGCAAGGACAATGACATTACAGGTGTTGAAGCGGTTATTGATAAGGACTTTACCGCAGCTAAGTTGGCCGAATTGGTC      4640
AAGCGTTCCTGTTACTGTAATGTCCACAACTTCGCCAATAACTATTCCTGAAATGGCGTCGATTCAACCGGCTTAACCAG

D  A  D  E  L  V  I  L  T  A  V  Q  Y  V  T  K  D  F  T  K  P  T  Q  E  D  I  K
GATGCTGATGAGTTAGTCATTCTAACGGCCGTGCAATACGTCACGAAGGACTTTACGAAGCCAACTCAGGAAGATATCAA      4720
CTACGACTACTCAATCAGTAAGATTGCCGGCACGTTATGAGGTGCTTCCTGAAATGCTTCGGTTGAGTCCTTCTATAGTT

 Q  A  T  V  A  E  M  Q  A  L  V  D  E  G  Q  F  P  A  G  S  M  K  P  K  V  E  A
GCAAGCAACGGTTGCCGAGATGCAAGCGTTGGTTGATGAAGGTCAATTCCCAGCTGGATCAATGAAGCCAAAGGTTGAGG      4800
CGTTCGTTGCCAACGGCTCTACGTTCGCAACCAACTACTTCCAGTTAAGGGTCGACCTAGTTACTTCGGTTTCCAACTCC

  A  M  S  F  V  T  A  T  G  R  N  A  V  I  T  S  L  D  N  I  A  A  Y  L  E  N
CAGCAATGAGCTTTGTTACTGCCACTGGTCGTAATGCAGTCATTACATCATTGGATAATATTGCAGCATATCTTGAAAAT      4880
GTCGTTACTCGAAACAATGACGGTGACCATCATTACGTCAGTAATGTAGTAACCTATTATAACGTCGTATCGAACTTTTA

G  D  G  T  V  I  T  A  *
GGGGATGGTACGGTAATTACCGCGTAATCCCAACGAAACAGACGGTTTCCGTATAAAGGAGTCCGTCTGTTTTTCATTTG      4960
CCCCTACCATGCCATTAATGGCGCATTAGGGTTGCTTTGTATGCCAAAGGCATATTTCCTCAGGCAGACAAAAAGTAAAC
                                                    wkaR⟶
                                                      M  L  Q  K  F  M  T  E
TGAAAGCGTATACTTATTAAAAGAAGAGTTTTCATAATGAACAAAAGGGGATTAGCATGCTACAGAAATTTATGACGGAA      5040
ACTTTCGCATATGAATAATTTTCTTCTCAAAAGTATTACTTGTTTTCCCCTAATCGTACGATGTCTTTAAATACTGCCTT

W  L  Y  I  S  D  Y  D  T  P  V  S  R  E  F  V  G  E  A  N  I  H  G  V  D  L  A
TGGCTGTATATTTCTGACTACGATACGCCGGTCAGTCGCGAGTTTGTCGGAGAAGCTAATATTCATGGTGTTGATTTAGC      5120
ACCGACATATAAAGACTGATGCTATGCGGCCAGTCAGCGCTCAAACAGCCTCTTCGATTATAAGTACCACAACTAAATCG

 K  Q  Y  V  A  V  V  V  K  G  E  T  A  T  M  P  E  Y  N  L  A  F  D  L  D  K  Y
GAAACAGTATGTGGCGGTTGTTGTTAAAGGGGAGACAGCGACCATGCCGGAATATAATTTAGCGTTTGATTTAGATAAAT      5200
CTTTGTCATACACCGCCAACAACAATTTCCCCTCTGTCGCTGGTACGGCCTTATATTAAATCGCAAACTAAATCTATTTA

  R  R  C  Y  I  M  T  D  E  C  V  P  S  F  T  S  A  L  A  D  S  F  S  I  G  I
ATCGGCGCTGCTATATCATGACGGACGAGTGTGTCCCGAGCTTTACAAGCGCTTTAGCCGATTCTTTTAGCATCGGTATT      5280
TAGCCGCGACGATATAGTACTGCCTGCTCACACAGGGCTCGAAATGTTCGCGAAATCGGCTAAGAAAATCGTGGCCATAA

G  T  A  H  F  D  I  S  A  S  V  K  E  A  L  Y  I  N  L  L  T  D  D  I  N  R  V
GGCACAGCGCACTTTGATATTTCGGCTAGTGTTAAGGAAGCCTTATATATCAATTTACTGACTGATGATATTAATCGGGT      5360
CCGTGTCGCGTGAAACTATAAAGCCGATCACAATTCCTTCGGAATATATAGTTAAATGACTGACTACTATAATTAGCCCA

 L  R  Y  N  K  F  A  Y  L  I  N  I  A  K  T  Q  S  A  D  K  N  I  V  A  Y  F  S
ATTGCGGTATAACAAGTTTGCCTATCTGATTAATATCGCCAAGACGCAGTCGGCAGACAAAAATATTGTTGCGTACTTTA      5440
TAACGCCATATTGTTCAAACGGATAGACTAATTATAGCGGTTCTGCGTCAGCCGTCTGTTTTTATAACAACGCATGAAAT
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  R  L  D  E  E  A  Q  L  T  L  W  I  Y  A  N  N  G  A  I  T  E  T  A  A  Q  L
GTAGGTTGGATGAAGAGGCGCAGCTGACTTTATGGATTTATGCCAATAATGGTGCCATTACGGAAACGGCGGCGCAACTG          5520
CATCCAACCTACTTCTCCGCGTCGACTGAAATACCTAAATACGGTTCTTACCACGGTAATGCCTTTGCCGCCGCGTTGAC

H  V  H  P  K  T  V  R  Y  R  L  N  K  V  M  A  T  T  G  L  D  P  K  V  G  V  D
CACGTGCACCCTAAAACGGTGCGTTATCGTTTGAATAAAGTAATGGCAACAACAGGATTGGATCCCAAGGTGGGAGTGGA          5600
GTGCACGTGGGATTTTGCCACGCAATAGCAAACTTATTTCATTACCGTTGTTGTCCTAACCTAGGGTTCCACCCTCACCT

 L  V  P  L  I  V  S  Y  I  Q  G  K  T  Q  D  Y  D  I  L  V  G  E  L  Q  S  I  S
TTTGGTGCCTCTTATCGTCAGTTACATTCAGGGGAAAACACAGGACTATGACATTCTAGTCGGTGAGTTACAGAGTATCT          5680
AAACCACGGAGAATAGCAGTCAATGTAAGTCCCCTTTTGTGTCCTGATACTGTAAGATCAGCCACTCAATGTCTCATAGA

  D  R  M  L  P  R  D  K  Q  A  H  *
CTGACCGTATGTTACCACGCGATAAGCAAGCCCATTAATCGGAACTGATGCAAAAAAG                          5738
GACTGGCATACAATGGTGCGCTATTCGTTCGGGTAATTAGCCTTGACTACGTTTTTTC
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As shown in Fig. 8, the ADI gene cluster, which was determined to be 5.7 kb long, consists of five-gene is encoded by arginine deiminase (*wkaA*), ornithine carbamoyltransferase (*wkaB*), the arginine–ornithine antiporter (*wkaD*), carbamate kinase (*wkaC*), and the putative transcriptional regulator (wkaR). The deduced amino acid sequence of WkaA has high identity with arginine deiminase (EC: 3.5.3.6), which is considered the key enzyme in the ADI pathway and converted the precursor arginine to intermediate citrulline. Based on the determined nucleotide sequence, the *wkaB* and *wkaC* genes were predicted to be ornithine carbamoyltransferase (EC: 2.1.3.3) and carbamate kinase (EC 2.7.2.2), respectively. The ornithine carbamoyltransferase catalyzes the conversion of citrulline to ornithine, whereas the carbamate kinase converts carbamoyl phosphate to ammonia and carbon dioxide. The determined sequence of *wkaD* gene is likely to play a role as an arginine–ornithine antiporter. The WkaR, is identified as a PucR family regulator protein, which is predicted to be a transcriptional activator. The PucR is a transcriptional regulator (mostly activator) involved in the catabolism of purine and its intermediates, such as uric acid, allantoin, or allantoic acid, and activates the purine degradation pathway.

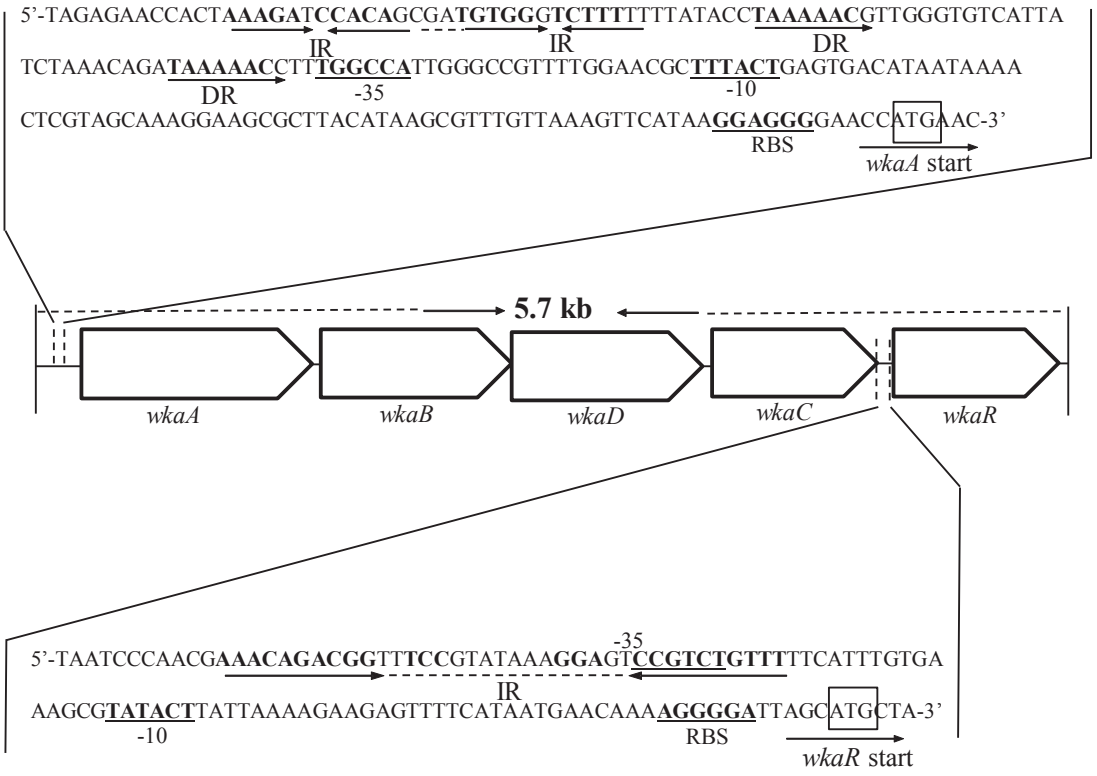

Fig. 8. Gene organization of the *wkaA-R* gene cluster in *W. confusa* K-28. The determined nucleotide sequence shows that the *wkaA* and the upstream region of *wkaA* contain an inverted repeat (13-38 bp) and two direct repeats composed of 48–54 bp or 79–85 bp. Latter nucleotide sequence contains *wkaR* and the upstream region of *wkaR*, which contains the inverted repeat unit composed of 4916–4952 bp.

By the RT-PCR analysis, the expression patterns of each ADI gene within the cluster during the fed-batch culture using a jar fermenter were evaluated (Fig. 9). From the RT-PCR data, it was confirmed that the expression level of all five-gene gradually decreased with the increase of cultivation time. In addition, the expression levels of those five genes did not differ in the presence or absence of the added fed-batch arginine,

revealing that the ornithine and citrulline production by the K-28 strain does not depend on the exogenous arginine.

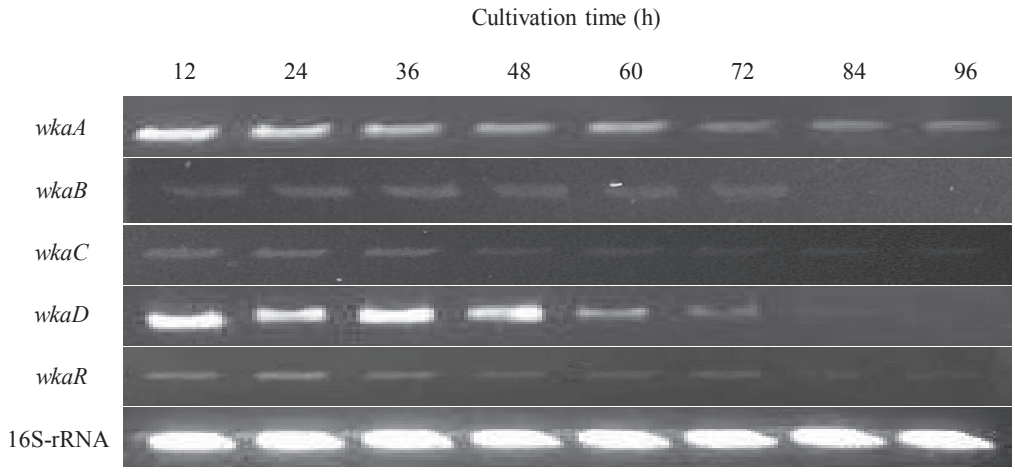

Fig. 9. Expression patterns of the *wkaA-R* gene during the consumption of arginine and the production of ornithine and citrulline. In the cultivation, a 3 L jar fermenter (working volume: 1-L) was used.

Effect of Aerobic or Anaerobic Condition on Ornithine Production

The expression level of w*kaA-R* genes in the ADI cluster was evaluated under the aerobic or anaerobic conditions (Fig. 10). The RT-PCR analysis shows that the expression of all five-gene in the ADI gene cluster is increased in the anaerobic condition compared with the aerobic one.

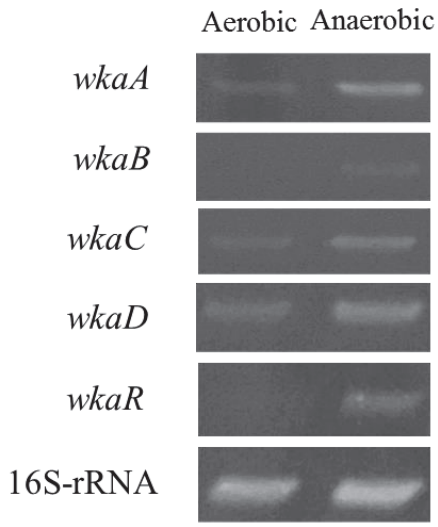

Fig. 10. Gene expression profile of *wkaA-R* genes in both aerobic and anaerobic conditions. In the figure, the left-side gene expression pattern indicates aerobic condition whereas the right-side expression pattern indicates anaerobic one. The cultivation was carried out by keeping the initial pH at 6.5 and temperature at 28°C with the supplemented 0.5% (w/v) arginine.

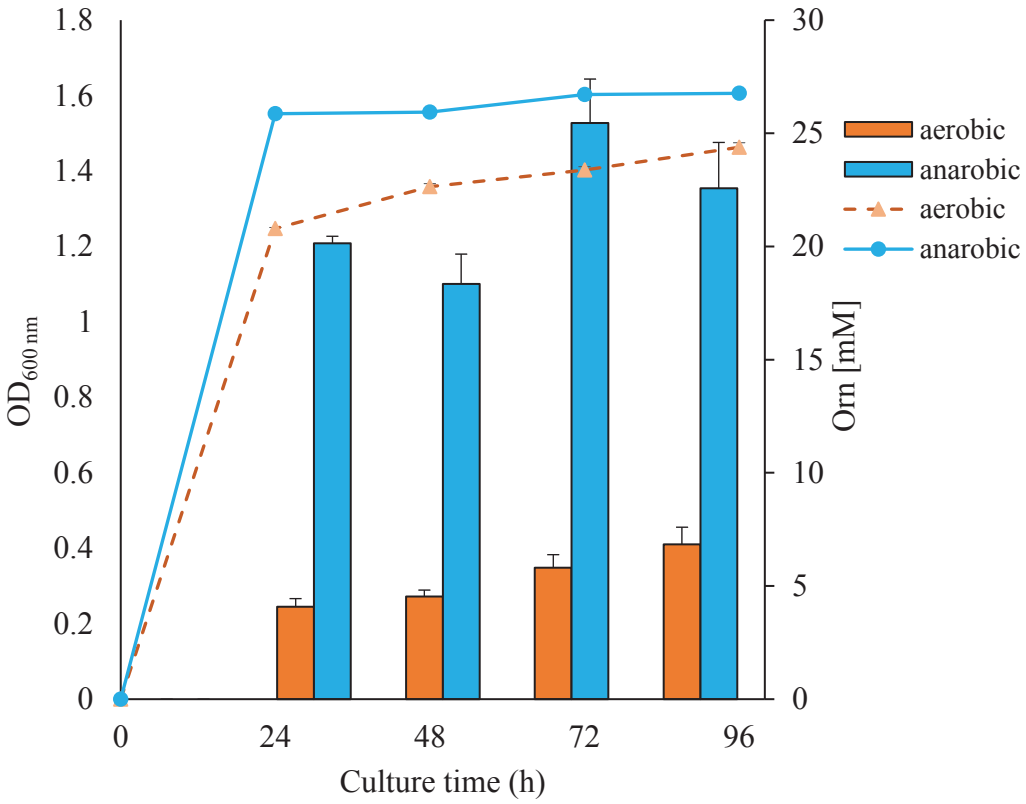

Fig. 11. Difference of ornithine production and cell growth under the aerobic or anaerobic condition. The cultivation was done at initial pH 6.5 and at 28°C using the medium supplemented with 0.5% (w/v) arginine. The data were expressed as mean ± SE (n=3).

The result suggests that ornithine production was significantly high under the anaerobic condition (20.2 ± 0.3 mM) at 24 h of cultivation (Fig. 11) but not under the aerobic condition (4.1 ± 0.4 mM ornithine).

ADI genes expression without or with arginine supplementation

Whether the ADI pathway induction is mediated by the exogenous arginine was also investigated. RT-PCR analysis suggested that, the expression of the five-gene did not differ in the presence or absence of arginine (Fig. 12).

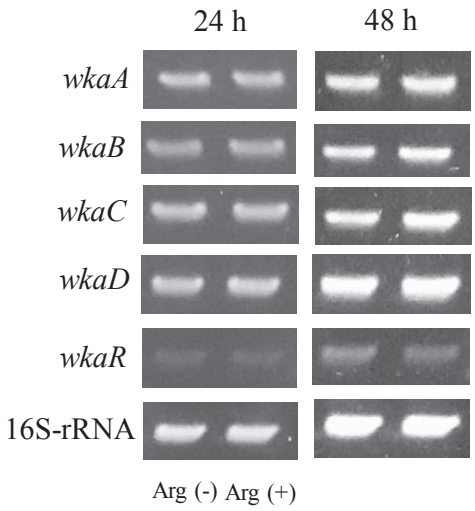

Fig. 12. Expression patterns of the ADI genes *wkaA-R* at 24 h and 48 h with 0% and 0.5% (w/v) arginine supplementation, where pH is maintained at 6.5 and temperature at 28°C during cultivation.

Ornithine high-production and Arginine Conversion in various Media

Since *W. confusa* K-28 is found to be safe as food materials from the acute oral toxicity and mutagenicity test, it was investigated whether the K-28 strain can produce ornithine or not. Various juice supplemented with 0.5% (w/v) arginine were used. From the chromatographic data, it was observed that the K-28-fermented carrot juice contained a high-level ornithine (Fig.13) with the high conversion ratio of arginine to ornithine (Fig.14), whereas the K-28-fermented pineapple juice did not contain ornithine at a high level. The other fermented juices were not suitable for high-ornithine production.

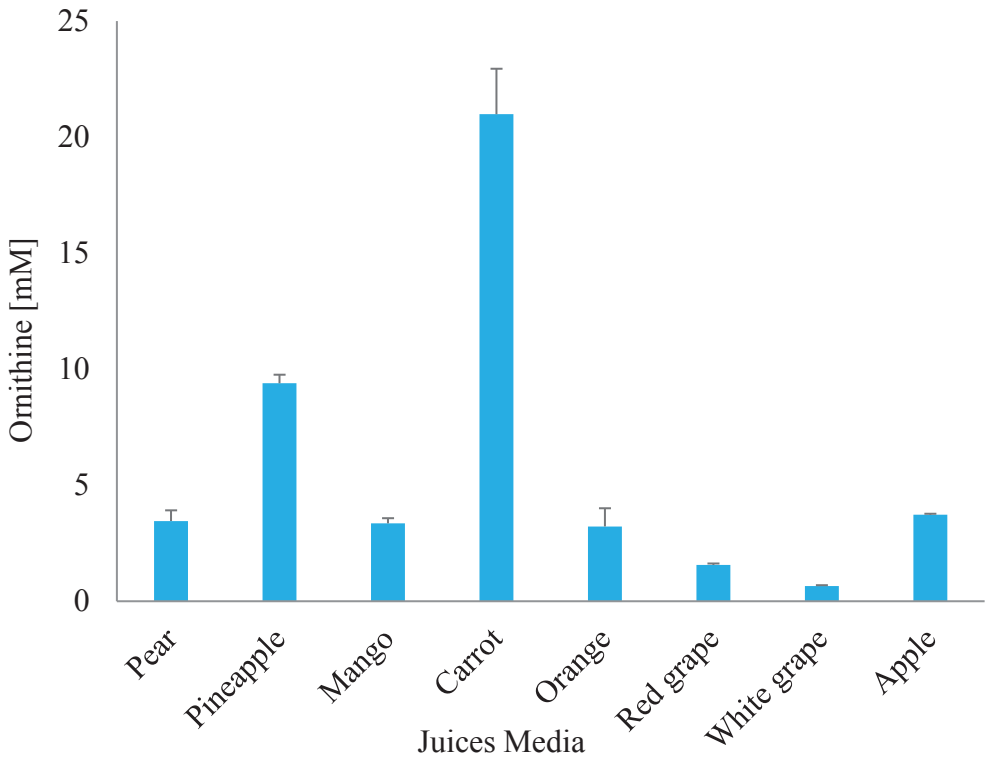

Fig. 13. Ornithine production on various juices media supplemented with 0.5% (w/v) initial arginine where fermentation was done by standing the initial pH 6.5 at 28°C. The data were expressed as mean ± SE (n=2).

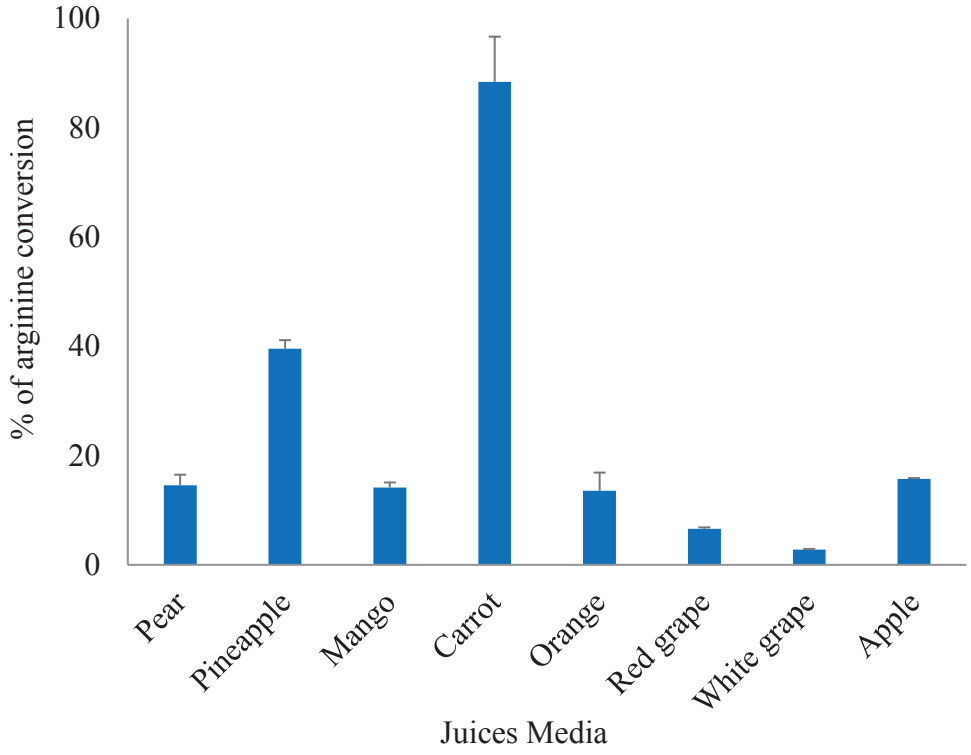

Fig. 14. Arginine conversion ratio to ornithine on various juices media (100%) supplemented with 0.5% (w/v) initial arginine by maintaining the initial pH 6.5 at 28°C. The data were expressed as mean ± SE (n=2).

DISCUSSIONS

Amino acids play a role as a source of energy to the nutritionally poor environment (Yu JJ and Oh SH, 2008). An acute toxicity test and a mutagenicity experiment indicate that, *W. confusa* K-28 is a safe and reliable bacterium. Interestingly, we observed that the K-28 strain produces high-level ornithine and intermediate amino acid citrulline with a complete conversion of precursor arginine. Since *W. confusa* K-28 was isolated from the flower of *Senna obtusifolia*, the strain might be adapted to environmentally harsh conditions. Moreover, the K-28 strain has high tolerance to gastric and bile acids (data not shown), suggesting that the plant-derived LAB strain may be effective to immuno-stimulation and improving constipation, like the plant-derived LAB strains isolated previously by us (Jin *et al.* 2010, Zhao *et al.* 2012). Therefore, *W. confusa* K-28 might be a probiotic strain which is useful for manufacturing functional food and healthcare supplements.

The level of L-ornithine produced by microbial fermentation does not reach to the industrial level. Although an endotoxin-free *Corynebacterium* (*C.*) *glutamicum* is used to produce industrially several amino acids, the endotoxin-producing *Corynebacterium* species is also present. The limited strains, which are the genetically modified mutants of *C. glutamicum*, have been claimed for ornithine production with satisfactory efficacy. In

compared to the mutant strains, the ornithine production by the wild-type *C. glutamicum* is only 0.5 g/L. In other words, the K-28 strain is superior to the wild-type *C. glutamicum* in terms of ornithine production and probiotic characteristics.

To produce ornithine, in general, the arginine catabolism occurred by arginase urease or arginine deiminase (Kuensch *et al.* 1974; Arena *et al.* 1999). In the present study, the author shows that *W. confusa* K-28, harboring the ADI pathway, has significant potential for ornithine production at a high level with a hyper conversion rate from arginine to ornithine. In the present study, it was observed that the K-28 strain produces a high-yield ornithine (67 ± 4 mM) *via* a continuous arginine-feeding method using a jar fermenter for 24 h (Table 4). As shown in Fig. 6, the ornithine production is fivefold greater than that *via* the laboratory-scale stand-cultivation method (12.7 ± 0.6 mM) for 24 h.

The ADI pathway, in general, consist of ADI, OTC, and CK enzymes, which are encoded by the *arcA*, *arcB*, and *arcC* genes, respectively (Remaux *et al.* 2012). These enzymes are known as the cytoplasmic enzymes and are called the skeleton of ADI pathway (Noens & Lolkema, 2017). Additionally, the presence of an arginine–ornithine antiporter, putative aminotransferases, and/or a transcriptional regulator have been reported by some bacteria which are encoded by the *arcD*, *arcT*, and *arcR* genes, respectively. The author found that *W. confusa* K-28 contain ADI cluster and enzymes

those are identical to ADI (*wkaA*), OTC (*wkaB*), CK (*wkaC*), the arginine–ornithine antiporter (*wkaD*), and the putative transcriptional regulator (*wkaR*).

L-citrulline is an amino acid which stimulates the muscle protein synthesis and the amino acid is a promising pharmaconutrient to provide nutritional support in malnourished patients of aging and have sarcopenia (Jourdan *et al.* 2015; Bahri *et al.* 2013). The amino acids have been reported to inhibit the high glucose–induced endothelial senescence in along or in combination with arginine (Tsuboi *et al.* 2018).

Arginine is known as a potential source of energy in the ADI pathway utilized by the bacteria, because it forms one molecule of ATP by the catabolism of per mole arginine at the time to produce the ornithine and ammonia (Tonon and Lonvaud-Funel, 2000). The ADI pathway has been considered as one of the functions of acid-base physiology within the bacteria (Casiano-Colón and Marquis, 1988). The increase of pH has been observed in *Streptococcus sangria*, due to the production of ammonia *via* the ADI cycle (Marquis *et al.* 1987).

In contrast, ammonia is a strongly basic molecule and it acts as a signaling molecule in yeast and ammonia oxidizing bacteria (Bollmann A & Revsbech NP, 2005; Plakova Z *et al*, 1997). Bacterial communities significantly changed during the fermentation which may reflect the production of various amino acids that decrease the pH of the fermented

medium. High arginine catabolism by the K-28 strain during the jar fermentation results in the formation of high-yield citrulline and ornithine with the concomitant production of ammonia and ATP (or energy). However, during the production of citrulline and ornithine with the concomitant production of ammonia by *W. confusa* K-28, the strain may cause the rising of pH, which could help the K-28 strain to survive in acidic environment. Moreover, since the K-28 strain produces both amino acids with over 100% conversion rate, the bacterium could survive longer than any other bacteria in acidic environment by utilization of the ADI system to increase the pH. The additional ATP production could help the bacterial growth to survive much longer than the bacteria that produce low level amino acids or did not use the ADI pathway.

In general, many heterofermentative LAB have the ability of producing energy *via* arginine catabolism by the formation of ornithine, ammonia, CO_2, and ATP (Garvie, 1967a; kandler & Weissi, 1986; Tonon & Lonvaud-Funel, 2002). It has been thought that most of the heterofermentative lactobacilli produce ammonia from arginine, whereas homofermentative ones and *Oenococcus oeni* do not produce ammonia in general. In addition, some heterofermentative lactobacilli have the ability of catalyzing first two-step (Step-1: 1 mole arginine -----> citrulline + 1 mole ammonia, and Step-2: citrulline -----> ornithine + carbamoyl phosphate), resulting in yield of only one molecule of ammonia.

To avoid the problem, it is recommended to use the supplemented arginine from 0.3% (w/v) to 0.6% (w/v). As the author used fed-arginine along with 0.5% (w/v) initial arginine in jar fermentation process, the author predicted to produce two-molecule ammonia (Step-3: carbamoyl phosphate -----> ammonia + ATP + CO_2) with the simultaneous production of ATP by *W. confusa* k-28, which ultimately results to survive the strain in acid sensitive environment.

In the human, citrulline is contributed as an intermediate of ureagenesis and contributed in nitric-oxide synthesis. Moreover, the metabolism of the orally administrated arginine is affected due to the expression of arginase (first-pass effect), resulting in poor oral bioavailability of orally administered arginine (Guoyao Wu, 1997; Castillo *et al.* 1993; Osullivan *et al.* 1998). Since citrulline is not the substrate of arginase, the amino acid can easily pass through the GIT and hepatic tissue. Therefore, the administration of citrulline may contribute to the increase of arginine oral bioavailability. Additionally, when arginine is orally administrated in combination with citrulline, the arginine can also pass through the GIT and hepatic tissue without undesirable metabolization by those organisms (Bahri e*t al.* 2013). This is because L-citrulline inhibits the arginase activity by acting as an allosteric inhibitor.

The production of ornithine gradually decreased with the increasing of cultivation time, although vigorous cell growth was observed at the time, whereas the citrulline accumulation was significantly increased. Due to equilibration between the substrate and product, the formation of citrulline from ornithine may be occurred, because the OTC enzyme catalyzes the reciprocal conversion between ornithine and citrulline. On the other hand, the ADI catalyzes the reaction only toward the citrulline anabolism. Therefore, at the time of fermentation, arginine is first converted to ornithine, and after the accumulation of the excess amount of ornithine, citrulline is started to produce in a gradual manner.

It has been reported that some LAB and other bacteria utilize the ADI pathway for arginine catabolism. The author compared the arginine catabolism and ornithine production by *W. confusa* K-28 with those reported by other strains. It has been reported by a research group that *W. koreensis* MS1-3 and MS1-14 produce extracellular ornithine at 45 mg/L and 46 mg/L respectively, when arginine was added with a final concentration of 1.0% (w/v). However, under the same culture conditions, *W. confusa* K-28 produced 8.85 g/L ornithine (Table 4).

In general, from the standpoint of food safety, when the MRS medium is used as a fermented base, the product cannot be administered orally, because MRS medium is a

laboratory medium and contains toxic compounds harmful to human health. Therefore, in the present study, various juices media supplemented with arginine were used as a culture media to generate ornithine. From the experimental data, when 100% (v/v) Carrot juice supplemented with 0.5% (w/v) arginine as a culture medium (pH 6.5) was employed, ornithine high production was observed at 28°C for 60 h. The ornithine production was 21 ± 2 mM (Fig. 13), with a conversion rate of 88 ± 8% (Fig. 14), suggesting that the plant-derived LAB, K-28 is suitable for growing in carrot juice.

The present study suggests that the expression of genes in the ADI cluster of *W. confusa* K-28 is enhanced at anaerobiosis than the aerobiosis (Fig. 10). The ornithine production from the exogenous arginine is induced under the anaerobic condition and it is predicted that the anaerobic induction may be mediated by some transcriptional regulators. The nucleotide sequence analysis of the ADI gene cluster (*wkaA–R*) in the K-28 strain suggests that there are two predicted transcriptional units, *wkaABDC* and *wkaR*. Moreover, the expression of these genes is likely to be regulated by some transcriptional regulator. The WkaR, which is predicted to be a transcriptional activator, has homology with a PucR family regulator protein. The PucR is a transcriptional regulator (mostly activator) involved in the purine catabolism and its intermediates, such as allantoin or allantoic acid, uric acid, and activates the purine degradation pathway.

In the study the author has found that, the expression levels of *wkaA–R* genes did not differ without or with arginine, and the resulted ornithine and citrulline production by *W. confusa* K-28 does not depend on the exogenous arginine. The K-28 strain produced ornithine and citrulline at a conversion rate of over 100%, suggesting that the K-28 utilizes arginine, which is predicted to produce by the proteins or peptides digestion in the medium.

The RT-PCR (Fig. 9,12) gene expression analysis suggested that, an increased arginine concentration (both initial and fed) did not enhance the expression of *wkaA–R*, the high production of ornithine by *W. confusa* K-28 correlated with the *wkaA–R* genes expressions, and an increased gene expression of *wkaA–R* in anaerobiosis (Fig.10) suggesting that WkaR may contribute as positive regulator under the anaerobic condition.

CONCLUSION

In the present study, the author isolated a total twenty LAB strains from several plant sources. After the isolation, the author tried to screen strain to produce ornithine and found that a LAB strain can produce ornithine when fermented with supplemented arginine in the MRS medium. After further investigation, it has been confirmed that the LAB strain produces ornithine a high level. In addition, the strain was found to produce citrulline, when cultured with the continuous arginine feeding. The LAB strain, designated as K-28, was identified as *Weissella confusa*. In the present study, the author confirmed that *Weissella confusa* K-28 produces ornithine and citrulline 18 ± 1 g/L and 10 ± 2 g/L, respectively, with a 100 ± 9% conversion rate when arginine was continuously fed into a jar fermenter.

Although, *Corynebacterium* (*C.*) *glutamicum* is industrially present for production of several amino acids, the endotoxin-producing other *Corynebacterium* species is also present. Since, the ornithine high producing *C. glutamicum* is also industrially present, but the strains have been genetically modified. In that consequence, the wild-type of *C. glutamicum* produces only 0.5 g/L ornithine, indicating that *W. confusa* K-28 is superior to *C. glutamicum* to use a probiotic microorganism.

The author investigated that whether the ornithine and citrulline production *via* the arginine catabolism by the K-28 strain is mediated by the ADI pathway or not. In addition, the author confirmed that *W. confusa* K-28 harbors an arginine deiminase (ADI) gene cluster which is 5.7 kb in length and harbors five-gene, namely *wkaA*, *wkaB*, *wkaC*, *wkaD*, and *wkaR*. The expression level of the five genes did not differ with or without arginine, suggesting that the production of both ornithine and citrulline in the K-28 strain was not induced by exogenous arginine. The production of ornithine and the expression of these five-gene *wkaA-R* significantly increases under the anaerobic condition than aerobic one, suggesting that the *wkaR* may contribute as a positive regulator under the anaerobic condition.

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