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# Effect of ammonium and amino acids on the growth of selected strains of *Gluconobacter* and *Acetobacter*

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ABSTRACT

Acetic acid bacteria (AAB) are a group of microorganisms highly used in the food industry. However, its use can be limited by the insufficient information known about the nutritional requirements of AAB for optimal growth. The aim of this work was to study the effects of different concentrations and sources of nitrogen on the growth of selected AAB strains and to establish which nitrogen source best encouraged their growth. Two strains of three species of AAB, *Gluconobacter japonicus*, *Gluconobacter oxydans* and *Acetobacter malorum*, were grown in three different media with diverse nitrogen concentrations (25, 50, 100, and 300 mg N/L and 1 g N/L) as a complete solution of amino acids and ammonium. With this experiment, the most favourable medium and the lowest nitrogen concentration beneficial for the growth of each strain was selected. Subsequently, under these conditions, single amino acids or ammonium were added to media individually to determine the best nitrogen sources for each AAB strain. The results showed that nitrogen requirements are highly dependent on the nitrogen source, the medium and the AAB strain. *Gluconobacter* strains were able to grow in the lowest nitrogen concentration tested (25 mg N/L); however, one of the *G. oxydans* strains and both *A. malorum* strains required a higher concentration of nitrogen (100–300 mg N/L) for optimal growth. In general, single nitrogen sources were not able to support the growth of these AAB strains as well as the complete solution of amino acids and ammonium.



## 1. Introduction

Acetic acid bacteria (AAB) are strictly aerobic microorganisms that are able to quickly and incompletely oxidize a large number of carbohydrates and alcohols, producing an accumulation of organic acids as the final products. This feature makes AAB useful for various biotechnological processes (Deppenmeier et al., 2002; Gullo and Giudici, 2008), such as the production of acetic acid from ethanol; gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from glucose; L-sorbose from D-sorbitol; and dihydroxyacetone from glycerol (Gupta et al., 2001; Lino et al., 2012; Prust et al., 2005). Nevertheless, the industrial exploitation of AAB is not fully developed (Mamlouk and Gullo, 2013), mainly due to problems with AAB recovery on solid media from extreme habitats and their high mutability (Mas et al., 2007). Furthermore, low cultivability could also be attributed to Viable But Not Culturable state that have been described for these microorganisms (Millet and Lonvaud-Funel, 2000; Torija et al., 2010). To solve the problem of low AAB culturability, different culture media have been developed to improve AAB isolation from different sources, with D-glucose, ethanol and/or mannitol as the carbon sources most widely used for the preparation of these enrichment media (Entani et al., 1985; Sokollek et al., 1998). However, relatively little new information has become available about the nitrogen and growth factor requirements in AAB since the studies about this topic conducted in the 1950s (Foda and Vaughn, 1953; Raghavendra Rao and Stokes, 1953; Rainbow and Mitson, 1953). Raghavendra Rao and Stokes (1953) reported that the growth factor requirements are critically influenced by the carbon and energy sources present in the medium. These authors also claimed the necessity of using peptone and yeast extract in culture media to ensure a sufficient supply of nitrogen for AAB growth. The problem of using these media is that there is no control over the nitrogen composition, and it is not possible to study AAB nitrogen requirements because the media are not chemically defined. Previously, Underkofler et al. (1943) reported that the use of a mixture of twenty amino acids can be used instead of hydrolysed casein for *Acetobacter suboxydans* growth, and the study also established pantothenic, nicotinic and *p*-aminobenzoic acids as the factors required for growth of this species. Later, Drysdale and Fleet (1988) suggested that most AAB are able to grow using inorganic ammonia as the sole source of nitrogen because they can synthesize all the amino acids from this compound; therefore, there are no essential amino acids for AAB. However, these authors also reported that some amino acids could have a stimulatory or inhibitory effect on the growth of some AAB species, and even earlier studies reported an essential role for some amino acids (Kerwar et al., 1964; Stokes and Larsen, 1945). Later, Trček and Teuber (2002) mentioned the difficulty to define an appropriate minimal medium for

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AAB growth because almost each strain of AAB has unique growth requirements. Finally, many new genera and species of AAB have been incorporated in the last years (Yamada et al., 2012; Trček and Barja, 2015), but only those growth data required for the description of these new species have been described.

In recent years, studies of nitrogen and AAB have mainly focused on ensuring that nitrogen was sufficiently available and appropriate for carrying out the acetification after alcoholic fermentation by yeast. For this reason, different studies have analysed the changes in amino acids during the production of vinegars from different types of raw materials and different acetification conditions (Álvarez-Cáliz et al., 2012; Callejón et al., 2008; Maestre et al., 2008; Valero et al., 2005). The effects of various physico-chemical operations, such as flocculation and filtration, during the stabilization of must and wines (Valero et al., 2005) and the biological ageing of wine (Álvarez-Cáliz et al., 2014) on the availability of nitrogen content for AAB growth have also been studied. Although all these practices are expected to decrease the concentrations of amino acids and vitamins available for the AAB growth, there are others, such as the autolysis of yeasts at the end of the alcoholic fermentation, that have the opposite effect and favour the growth of AAB (Fleet, 2001). However, extreme media, such as wine with a low pH and a high ethanol concentration, could also modify the amino acid requirements of AAB, increasing their nutritional demand (Drysdale and Fleet, 1988). All these studies have demonstrated that AAB growth depends on the substrate used. In the case of wine vinegars, grape musts are rich in arginine and proline; moreover, the latter cannot be used by yeast (Ribéreau-Gayon et al., 2006) and is the major amino acid in wines and one of the amino acids most used by AAB (Álvarez-Cáliz et al., 2012; Callejón et al., 2008; Maestre et al., 2008). Other substrates, such as ethanol or cider, are clearly nitrogen-poor, resulting in the need to add nutrients to favour AAB growth. Therefore, the concentration and type of nitrogen sources available for AAB growth could be a limiting factor for the best development of a specific process.

In a previous study (Sainz et al., 2016), three wild AAB strains were selected for the production of a new strawberry beverage, which was based on the production of D-gluconic acid from D-glucose to maintain the natural fructose from the strawberries in the final product. Two of these strains belong to the *Gluconobacter* genus: CECT 8443, a strain of *Gluconobacter japonicus* isolated from grape must (Navarro et al., 2013) and Po5, a strain of *Gluconobacter oxydans* isolated from wine vinegar (Vegas et al., 2010). The other strain, CECT 7742, belonging to *Acetobacter malorum*, was the only strain isolated from strawberry vinegar (Hidalgo et al., 2013).

Hence, the main aim of this work was to determine which nitrogen sources were the best for the growth of the selected strains and what was the minimum concentration needed to promote its growth. We determined the nitrogen requirements of these three wild strains, and used the type strain of each species for comparison. For that reason, we first analysed the growth of the six strains in different culture media using a range of nitrogen concentrations to establish the minimum nitrogen concentration for the optimal growth of each strain. Afterwards, in the optimal medium with minimum nitrogen concentration, individual amino acids or ammonium were added to determine the best nitrogen source for each strain.

## 2. Materials and methods

### 2.1. Microorganisms

Two strains from three different species (*G. japonicus*, *G. oxydans* and *A. malorum*) of AAB were used in the study (Table 1). All the strains were initially grown in GY liquid media (5% (*w*/*v*) D-glucose and 1% (*w*/*v*) yeast extract; Panreac, Barcelona, Spain) at 28 °C with shaking (125 rpm).

**Table 1**
Strains used in this study.

| Species | Strain | Origin or isolation source | References |
|---|---|---|---|
| *G. japonicus* | LMG 1373$^T$ | *Myrica rubra* | Malimas et al. (2009) |
| | CECT 8443 | Grape must | Navarro et al. (2013) |
| *G. oxydans* | 621H | – | De Ley (1961) |
| | Po5 | Vinegar | Vegas et al. (2010) |
| *A. malorum* | DSM 14337$^T$ | Rotting apple | Cleenwerck et al. (2002) |
| | CECT 7742[a] | Strawberry vinegar | Hidalgo et al. (2013) |

T: type strain.
[a] This strain has been incorrectly referenced as CECT 7749 in previous studies (Hidalgo et al., 2013 and Sainz et al., 2016).

### 2.2. Determination of nitrogen requirements

#### 2.2.1. Media used

The effect of the nitrogen source on the growth of the strains was tested in three different media: synthetic medium (SM) prepared according to Riou et al. (1997); yeast nitrogen base medium (YNB; yeast nitrogen base without amino acids (Becton Dickinson & Co, Franklin Lakes, NJ, USA)); and M9 minimal medium (Harwood and Cutting, 1990). For the preparation of M9, a concentrated salt solution (5×) with 64 g/L sodium hydrogen phosphate heptahydrate, 15 g/L monopotassium phosphate and 2.5 g/L sodium chloride was first prepared for a stock solution. Then, to prepare 1 L of the M9 media, 200 mL of this concentrated salt solution was mixed with 2 mL magnesium sulfate (1 M), 0.1 mL calcium chloride (1 M), and different nutrient solutions (sugar, nitrogen, vitamins) and brought to 1 L with distilled water. The three media tested had an initial sugar concentration of 5% (*w*/*v*), composed by equimolar concentrations of D-glucose and D-fructose, and 10 mL/L vitamins (100×) and 1 mL/L oligo elements (1000×) were added to each medium. The concentrated solution of vitamins (100×) was prepared with 2 g/L myo-inositol; 0.15 g/L calcium pantothenate; 0.025 g/L thiamine hydrochloride; 0.2 g/L nicotinic acid; 0.025 g/L pyridoxine; and 3 mL biotin (100 mg/L). The oligo elements solution (1000×) was comprised of 4 g/L manganese sulfate monohydrate; 4 g/L zinc sulfate heptahydrate; 1 g/L copper sulfate pentahydrate; 1 g/L potassium iodide; 0.4 g/L cobalt chloride hexahydrate; 1 g/L boric acid; and 1 g/L ammonium heptamolybdate.

Different nitrogen concentrations (25, 50, 100, 300 mg N/L and 1 g N/L) were added to media, initially as a complete solution of ammonium and amino acids, taking into consideration all the nitrogen atoms. When the optimal nitrogen concentration was determined for each strain, all the nitrogen was added as a single amino acid or ammonium ions to establish the best nitrogen source for each strain.

Amino acids solutions have been prepared with distilled water at a concentration of 2.5 g N/L and filtered. The amino acids used were: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); phenylalanine (Phe); γ-Aminobutyric acid (Gaba); glycine (Gly); glutamic acid (Glu); glutamine (Gln); histidine (His); isoleucine (Ile); leucine (Leu); lysine (Lys); methionine (Met); ornithine (Orn); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp) and valine (Val). Gaba was included in the study because although this compound is not an alpha amino acid, it is present in natural musts and we were trying to mimic these musts. Ammonium was always used in form of ammonium chloride.

All the chemicals were from Sigma Aldrich (Germany).

#### 2.2.2. Growth monitoring

For all experiments, the initial culture had optical density (OD; 600 nm) of ca. 0.1. Assays were performed using a microplate reader SpectroStar Nano (BMG LABTECH) at 28 °C in triplicate. The absorbance was measured every 30 min for 100 h, that is, 200 measurements, with stirring at 500 rpm for 80 s prior to each reading. For the representation of the growth the OD readings were normalized by dividing each value