Alternative nutrient sources for biotechnological use of *Sporosarcina pasteurii*

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Abstract
Microbial induced calcium carbonate precipitation (MICCP) is a process with great possible applications for ground improvement, consolidation of building structures and ornamental stone, or in developing the competitive bio-materials in the building materials field. Due to its lack of pathogeny and its great capacity to produce high amounts of carbonate in short time via urea hydrolysis, *Sporosarcina pasteurii* is one of the best candidates for obtaining the bacterial calcium carbonate in the presence of free calcium. The use of affordable ingredients besides the guarantee of optimal growing conditions in the same time, are the main requests for producing the bacterial biomass for industrial purposes. The industrial wastes coming from the dairy and brewery industries, as well as different types of manures, from poultry industries and fertilizer urea, were tested as alternatives sources for developing the ureolytic active bacteria *S. pasteurii*. Diary wastes such as whey and buttermilk are potential alternative sources for the bacteria development, while fertilizer suits perfectly as ecological (educt) substitute for commercial urea.

Keywords (4-6)
*Sporosarcina pasteurii*, bioremediation, microbial induced calcite precipitation, ecocementation, industrial effluents, waste management

Introduction
The Microbial Induced Calcium Carbonate Precipitation (MICCP) via urea hydrolysis has been used by many researchers because the ureolytic bacteria are widespread in the environment (Fujita et al. 2000) and are bearing the most efficient way for carbonate generating process that can be easily controlled (Hammad et al. 2013). In this way, high concentration of carbonate within a short period of time could be generated. The MICCP involves a series of complex biochemical reactions (Stocks-Fischer et al. 1999; Achal et al. 2011; Li et al. 2011), in which urease, and recently found carbonic anhydrase (Achal et al. 2011) are key enzymes in the biologically induced calcification process. The hydrolysis of urea is catalysed by means of the enzyme urease. As a consequence, urea is degraded to carbonate and ammonium, resulting in an increase of pH and carbonate concentration in the bacterial environment, which induces the formation of CaCO₃ in the presence of free calcium (Stocks-Fischer et al. 1999). Factors such as type of bacteria, bacteria cells concentration, temperature, urea concentration, calcium concentration, ionic strength, and the pH of the media may have a significant impact on the MICCP, and therefore on the microbial enzymatic activity. Large amounts of food wastes result from the production, preparation and consumption of food, which pose increasing disposal and pollution problems. If they could be effectively utilized, they indeed would be a valuable ecological source of nutrients, due to these large amounts of organic material (proteins, carbohydrates, lipids). The *S. pasteurii* nutritional profile is based on proteins (Whiffin, 2004 thesis). Only few alternative protein sources were tested and proposed till now for developing *S. pasteurii* biomass. The alternative sources containing non-lysed cells (brewery waste yeast and sludge biomass from waste water treatment processing) were inaccessible for the growing of *S. pasteurii*, while within the tested pre-lysed protein sources, such as corn steep liquor, *Torula* yeast and Vegemite, only the latter one showed high levels of urease activity compared to standard nutrient media (CASO, NAR) (Whiffin, 2004), used by itself or partially replaced with acetate. Another potential alternative nutrient source was found to be lactose mother liquor (LML), an industrial effluent of the dairy industry, and was proposed as a substitute to the standard media (Achal et al. 2009).

This paper aims to compare and test different alternative nutrient sources coming from dairy and brewery industries (including wastewater from a dairy factory), as well as different types of manures (from poultry industries and fertilizer urea) for the ureolytic active bacteria *S. pasteurii*. 
The alternative nutrients from industrial waste streams differ slightly in their composition even if they are from the same plant. For this reason, we decided to test LML, brewery yeast and Vegemite™ as alternative nutrients in reference like reported elsewhere (Whiffin, 2004, Al-Thawadi, 2008). For the sake of evaluating the natural variance of the waste stream nutrients, we compared our results also with the suggested and investigated nutrients reported by Whiffin 2004, Al-Thawadi 2008 and Achal2009. The efficiency of these media have been evaluated by conductivity measurements, pH, optical density, ammonium concentration evaluations in bioreactor trials, as well as by determining the vitality of the biomass through ATP measurements.

**Materials and methods**

**Biological material**

*Sporosarcina pasteurii*, 33 type strain, was purchased from the DSMZ (German collection for microorganisms and cell cultures). It was grown aseptically under batch cultivation conditions according to the DSMZ instructions and stored on agar plates at 4°C in the fridge, and used when necessary. All the experiments were performed in the same way, following a protocol defined as *standard bacterial inoculum*, starting from a stock culture not older than one month, in two revitalization (R) steps: R1 – a loop containing bacterial biomass from stock culture inoculated in 20 mL standard nutrient medium (SNM) containing 20g/L urea (incubated 8 h), and R2 – 0.5 ml of R1 in 100 ml of SNM with urea (incubated overnight) at 25°C-30°C and 150rpm. In the morning the R2 should be in the optimal condition for its experimental use - late exponential phase or in the early stationary phase, with values for the OD$_{600}$ of about 1.900 – 2.000 rau and the ATP values about 400 000 rlu/20 µL of R2 culture. For any type of experiments, the inoculum should be about 4-5 ml of the R2 inoculum for 100 ml of new nutrient medium (NM), in order to have an initial OD$_{600}$ of about 0.1000 rau.

**Urea and nutrient substitutes**

The SNM is based on CASO broth or NAR (both 8g/L of protein concentration) and 20 g/L of pure grade urea (SU) from Sigma Aldrich. The protein concentration of each nutrient substitutes was adjusted to 0.8 %, while the less rich in proteins ones were used as such. The nutrient substitutes such as different dairy products (whey, buttermilk, lactose mother liquor and wastewater from dairy industry), wastes from brewery industries containing yeasts, some commercial products (bakery yeast, brewery yeast, Vegemite) and the urea substitutes, are reported in Table 1. Part of these products were purchased by SOLINTEL, while other were obtained by the Fraunhofer IFAM or ICVBC from a local structures (farm, factory). The elemental composition of poultry manures (Table 1) was determined using an elemental analyzer NA 1500 Series 2, Carlo Erba Instruments.

### Table 1: Overview of investigated nutrient substitutions

<table>
<thead>
<tr>
<th>Class of substitutes</th>
<th>Alternative substitutes</th>
<th>Name</th>
<th>Characteristics* (A, B, C, D)</th>
<th>Source</th>
<th>Experimental use</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Urea substitutes</td>
<td>Exsiccated fertilizers based on poultry manure, used as organic fertilizers</td>
<td>PAV</td>
<td>3.8 (A) 32.75 (B) 29.34 (C)</td>
<td>a final product from the FERPODE Project - AMEK, Ferrara, Italy</td>
<td>Not filtered, 222 g/L**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP1</td>
<td>4.86 (A) 28.93 (B) 24.05 (C)</td>
<td>AgrofertiliSoc. Coop. A.R.I., Santa Sofia – Forlì, Italy</td>
<td>Not filtered, 266 g/L**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP2</td>
<td>2.82 (A) 30.36 (B) 23.56(C)</td>
<td>AL.FE S.r.l., Pomponesco, Italy</td>
<td>Not filtered, 233 g/L**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PHX</td>
<td>5.28 (A) 20.19 (B) 18.14 (C)</td>
<td>ITALPOLLINA, Rivoli Veronese, Italy</td>
<td>Not filtered, 156 g/L**</td>
</tr>
<tr>
<td></td>
<td>Fertilizer urea</td>
<td>FU</td>
<td>48.97 (A) 19.84 (B) 18.89 (C)</td>
<td>AL.FE S.r.l., Pomponesco, Italy</td>
<td>Filtered and not filtered, 20 g/L</td>
</tr>
<tr>
<td>B. Nutrient substitutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dairy products</td>
<td>Buttermilk (Mazada)</td>
<td>M</td>
<td>3 (D)</td>
<td>COVAP, Spain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactose Mother Liquor (LML)</td>
<td>LML</td>
<td>33 (D)</td>
<td>COVAP, Spain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey (Permeate)</td>
<td>W1</td>
<td>&lt; 1 (D)</td>
<td>COVAP, Spain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>W2</td>
<td>&lt; 1 (D)</td>
<td>From a farm in Oyten, Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>W3</td>
<td>&lt; 1 (D)</td>
<td>MUKKI LATTE, Italy</td>
</tr>
</tbody>
</table>
**Urease activity and biomass evolution**

The enzyme activity of urease was calculated for conductivity measurement at 30°C in the presence of 1 M urea by the use of urease TYPE IX from jack beans (Sigma Aldrich) as a standard. The conductivity measurement is one of the best and easiest methods for measuring the urease activity, because urease turns the urea molecule (not conductive) into two charged ions: ammonium (NH₄⁺, positively charged) and carbonate (CO₃²⁻, negatively charged). The following instrumentation and methods were used: OAKTON ion selective electrode (NH₄⁺), M2M sensor or Hanna DIST® Tester, DIST 6 (conductivity) and Nessler method (total ammonia). The biomass development was checked taking into account other parameters such as pH (Hamilton sensor, O₂ (Oxygens 120), optical density OD₅₆₀ (Beckman DU 640 Spectrophotometer), ATP (3M Luminometer and clean trace probes) and CFU (Colonies Forming Unit). The standard curves and the conversion factors between these analytical methods were elaborated and are shown in the SUPPLEMENTARY MATERIAL.

**Results**

Typical behavior of *S. pasteurii*

The typical behavior of a liquid culture of *S. pasteurii* (Fig. 1a-b) leads to significant changes in the initial nutrient medium during the bacterial growth. The initial pH of the nutrient solution (around 7) slightly increases toward an alkaline pH (9.2 – 9.4). The conductivity of the solution and the level of the ammonium ions increase in time, as a result of the enzymatic reaction. The maximum of pH is reached when the ionic equilibrium is established. The medium acts as a buffer and the bacterial culture is in a quite comfortable level of pH. During the bacterial culture growth, the initial oxygen present in the nutrient medium seems to decrease in time, until it is completely consumed. After about 10h, the anaerobic conditions start to prevail, the bacteria being in the stationary phase still present (Stacy L. Parks 2009). The urease activity of 1mL *S. pasteurii* working culture (30°C) corresponds to about 0.23 mg/mL urease TYPE IX from jack beans that hydrolyzes about 2 mM urea/min. Assuming the turnover rate of urease TYPE IX this should correspond to 100 -150 μmol NH₃/min (Fig 1c).

![Fig 1](image-url) Typical behavior of the bio enzymatic reaction parameters inside the Bioreactor (2 liters): (a) Conductivity, Oxygen, pH and Ammonium; (b) Optical Density and ATP and (c) the bacterial enzymatic activity compared with three different concentrations of pureurease. The initial biomass of *S. pasteurii* was 0.0079 for OD₆₀₀ and 15334 RLU/mL for ATP. The nutrient medium was CASO (30g/L) with fertilizer urea (0,3 M) at 24°C (a, b) and 30°C (c).

**Influence of the urea substitutes**

Compared with expensive pure grade urea (SU), the fertilizer urea (FU) showed the same behavior in the presence of the commercial urease TYPE IX from jack beans(Fig. 2a). No significant difference was observed between the two types of urea as regard the enzymatic activity during the bacterial development as well. The urease activity showed the
same trend in time for both types of urea tested (Fig. 2b). The maximum urease activity was observed in the exponential phase, with a maximum ureolytic rate of 0.0829 mS/cm/min for the pure grade urea (SU) and 0.0866 mS/cm/min for the fertilizer urea (FU). The conductivity and ammonium values increased in time in the same manner for both types of urea tested. The conductivity as well as the biomass increase exponential after a short time of adjustment as ATP measurements indicated (not shown).

Compared with expensive urea purchased from Sigma (SU), fertilizer urea (FU) had no negative influence in the recorded growth curves of S. pasteurii. Therefore a substitution of SU with FU is recommended to reduce the production costs, but the environmental impact of urea production should be considered as well. For this reason, possible urea substitutes derived from poultry manure were taken into consideration and were investigated (Table 1).

The type of available nitrogen (organic and/or ammonia) present in the exsiccated poultry manures tested, induced only a slightly enzymatic activity of the S. pasteurii, higher for PAV and lower for the other ones, with respect to the standard nutrient medium (Fig. 3a). If the FU is added to these media, even in small amounts (25% with respect to the urea present in the reference), the enzymatic activity significant increased, reaching an enzymatic activity of 76% for PAV and of 62% for EP1 with respect to the reference (Fig. 3b and Fig. 4b).

From Fig. 3 (a,b) the bacteria seems to behave best in the presence of EP2 (EP-Al.Fe.). EP2 indicates an even better performance supplemented with urea (with respect to EP1), when the change of conductivity parameter is considered. But the total ammonia parameter, showed that bacteria has no enzymatic activity in the presence of EP2 without urea (Fig. 4a). In fact, the EP2 with bacteria and EP2 without bacteria gave the same results (Fig. 5a). This medium might conserve the S. pasteurii inactive, which became enzymatically active when supplemented with urea.
Fig. 4. Total ammonia (NH$_4$-N) released due to the metabolic activity of the S. pasteurii in the presence of the urea substitutes nutrient mediums without (a), and with supplemental urea (b) for 24 h.

The results of the total ammonium release for all substrates demonstrate, that conductivity measurements are not sufficient for the evaluation of the S. pasteurii activity. The PHX fertilizer itself solubilizes many ions in the water solution (Fig. 5b), as the initial conductivity is very high (about 64 mS/cm). However, the bacterial urease activity was detected for this solution (Fig. 3, Fig. 4): if urea was added in a diluted solution, the conductivity as well as the total ammonia values changed per minute. This could indicate that inhibitory concentration of available N are present in the solution, or the presence of the other ion types, such phosphates, inhibits the bacterial enzymatic activity.

Fig. 5. The total ammonia changes in time of the EP2 (a) and the conductivity changes in time of the PHX urea substitutes (b) in the presence and absence of S. pasteurii.

The bacterial biomass increased very slow and had a lag phase of about 3 hours for media containing the urea substitutes compared to the reference (CASO). The bacteria passed already the exponential phase in that time. In media with PAV, the biomass reached the value of consistency (measured with the ATP) in about 20 hours, by maintaining an active growth phase. But therefore overcoming that one of the reference, in which the nutrients and urea were completely depleted and the bacteria had been in the decline phase. This indicates that nutrients are present in the tested media with substitutes. The best results in terms of bacterial growth of the tested potential urea substitutes containing no additional urea was observed for PAV (Fig. 6).

Fig 6. Biomass development in time expressed as ATP in the presence of different urea substitutes nutrient sources.
The comparison between the sterile and non-sterile organic fertilizers showed that, beyond *S. pasteurii* other bacteria are present in the non-sterilized PAV, EP1, EP2 and PHX. But in every tested substitute, activity of *S. pasteurii* was detected.

The initial pH of alternative media is a bit different than CASO (Fig. 7), more acidic for EP1st and PHX, and more alkaline for EP1 and EP2. The pH increased with the biomass uptake of *S. pasteurii* until the most favorable pH of this bacteria (about 9.4), especially in the samples containing the EP or PAV organic fertilizers when mixed with urea. Based on these results, it seems that this material cannot be used as an urea substitute, but it was observed that some of the tested dehydrated poultry manures kept the bacteria vital, that they might be partly appropriate as nutrient source.

**Fig. 7.** The pH changing in time in the presence (continuous and large dashed lines) or absence of *S. pasteurii* (small dashed lines) for the urea substitutes (EP1, EP2, PAV, PHX and CASO reference), with (continuous lines) or without (large dashed lines) additional urea.

**Influence of protein content**

The determination of the bacterial enzymatic activity was recorded using the reference nutrient medium CASO (20 g/L according to the provider protocol) and two lower concentrations (5 g/L and 8 g/L) with an urea concentration of 20 g/L. The results showed the expected conductivity increase for the 2% and 0.8% protein concentration, while for the lowest one (0.5%), the bacteria seems not to be enzymatically active in the lag phase, starting to hydrolyze the most of the urea with about two hours of delay with respect to the other two more concentrated media (Fig. 8). In fact, the bacterial enzymatic activity for 2% and 0.8% protein concentration is very similar in the exponential phase (between 2 and 4h), with values of the urease activity of 0.1439 and 0.1400 mS/cm/min. For the same interval, the bacterial urease activity in the 0.5% protein concentration nutrient medium is with only 25% less, with a value of 0.1075 mS/cm/min. After 6h, the beginning of the stationary phase is already started for bacterial biomass grown in the two highest protein concentration, with a drastic decrease of the urease activity (0.02933 and 0.0166 mS/cm/min), while in the case 0.5% protein concentration, the bacteria still have a good enzymatic activity (0.0659 mS/cm/min) for the 2% and 0.8% protein concentration.

**Fig. 8.** Conductivity (dashed lines) and ammonium (continuous lines) changeover time of *S. pasteurii* bacterial culture grown with 2%, 0.8% or 0.5% protein concentration (a) and the bacterial biomass development (b).
The bacterial activity in the alternative nutrient media showed an increase of conductivity over time for almost all tested nutrient substitutes (Fig. 9). The dairy (buttermilk M, whey W1-W3 and waste water wH2O) and liquid brewery yeast products (Be1) were distinguished between sterile (st) and non-sterile in order to investigate the influence of the present competing bacteria/yeasts. The pH was adjusted in advance to 7 in case of the acidic nutrient substitutes (LML, M and W1-W3). It was assumed that enzymes from the dairy nutrients were still active and competing with the urease off S. pasteurii. It was observed, that once the alkaline conditions were reached, the ‘dairy enzymes’ could no longer compete with urease. The dairy product whey had higher urease activities than buttermilk no matter if sterile or not, and even better than the yeast based sources. Within the three types of whey tested, the W3 was preferred by the S. pasteurii. The initial alkali pH of the wH2O favored the growth of biomass (Fig. 10) and therefore the amount of urease (Fig. 9), the waste water coming from the cleaning system (Mukki Latte) gave satisfactory results as well. The bacteria displayed a quite constant enzymatic activity in the presence of LML, with a maximum of 0.033mS/cm/min after 6h, similar to V and NAR.

![Fig. 9](image)

**Fig. 9.** The bacterial enzymatic activity of S. pasteurii in low protein content nutrient media (legend, see table 1).

S. pasteurii showed the typical growth curve only in the presence of CASO and NAR nutrient medium. The tested potential nutrient sources tested as substitutes had an influence the bacterial vitality (Fig. 10). The behavior of S. pasteurii in the CASO nutrient medium (0.5 %) was similar to the ones of the dairy alternative nutrient media with a protein content < 1 (see table 1). The main difference was the longer lasting lag phase of the bacterial growth for all the tested nutrient substitutes. The lowest enzymatic activity was observed for brewery based alternative nutrients. But a high number of vital cells was attributed to the brewery yeasts as well. In this case it seems that the presence of the competitors (brewery yeast) and maybe traces of ethanol, inhibited the ureolytic bacteria growth (Fig. 23-24).

![Fig. 10](image)

**Fig. 10.** Biomass growth over time expressed by ATP (rlu/mL) in the presence of different alternative nutrient sources.

**Conclusion**

The most effective alternative urea substitute is Fertilizer Urea. The tested poultry manure for this aim contained too little of available Nin urea form, the performance of bacterial enzymatic activity being therefore limited with respect to the reference (Fig. 11). But the experimental data show that this matter contains high amounts of organic substances.
that could be suitable as nutrient substitutes. The bacterial growth with poultry manure seems to come along with less urea and similar enzymatic activities as the standard nutrient medium.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>NH₄-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>PAV</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>EP1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EP2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>PHX</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

Fig. 11. The performance of bacterial enzymatic activity in the presence of fertilizer urea (Ref) and different types of urea substitutes.

Within the possible alternative nutrient sources for the grow of *S. pasteurii* ureolytic bacteria, the byproducts coming from dairy industry gave the best results with respect to yeast based products coming from brewery or bakery industry. Whey, called also permeate, followed by the alkaline water ensued after the cleaning of the installation system of the dairy factories and the LML are potential candidates for the use as a cheap and environmental-friendly nutrient source for biotechnology. Whey (Permeate) originated from dairy waste industry with about 80% of the enzymatic activity with respect to the CASO medium (REF), but with a longer lag phase in the bacteria growth. In fact, taking into account, that Sporosarcina pasteurii was constantly maintained in CASO nutrient medium, these bacteria need previously to be accommodate with the new alternative nutrient source. Therefore, for any further experiments it is necessary to employ the biomass (R1 and R2), using SP adapted to the new nutrient source.

**Acknowledgements**

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**References**


**Figure Captions**

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
• Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
• Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

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• For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.
• For books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.
Correspondence between the $\text{NH}_4^+$ readings by the sensor and the values of $\text{NH}_3\text{-N}$ obtained using the Nessler method

Different concentrations of $\text{NH}_4\text{Cl}$ (marked by ⬤) containing the ionic strength adjuster (NaCl 5M) were measured by the two methods. Concentrations tested were: 35.70 mM (500 ppm NH$_4$ as N); 71.40 mM (1000 ppm); 357.00 mM (5000 ppm); 535.50 mM (7500 ppm); 714.00 mM (10000 ppm) and 1071.42 mM (15000 ppm). For the Nessler method the solutions were diluted as described in ??? (publication). A linear relationship was observed, with higher values for the chemical assessment.

![Figure A2. Correlation between the two types of measurements (Nessler method and the selective ion sensor measurement), using ammonium standard solutions used for the OAKTON electrode calibration.](image-url)
APPENDIX B

B1 - Conversion of NH\textsubscript{3}-N and NH\textsubscript{4}\textsuperscript{+} to urea hydrolysed

Standard curves of ammonium and total ammonia concentration resulting from complete hydrolysis of several concentration of urea by purified urease (Urease Type IX from Canavaliaensiformis, Sigma), at 30°C, were generated. Two different types of urea were tested: the pure grade urea (Sigma) and fertilizer urea, commonly used in the agriculture. The amount of total ammonia (Nessler method) and NH\textsubscript{4}\textsuperscript{+} ions were measured at the end of hydrolysis, when the reaction of urea hydrolysis was complete.

![Diagram](image)

Figure B1.1. Standard curves of ammonium and NH\textsubscript{3}-N registered at the complete hydrolysis of different concentration of pure urea (Sigma grade).
Figure B1.2. Standard curves of ammonium and NH$_3$-N registered at the complete hydrolysis of different concentration of fertilizer urea.

From these figures, the following relationship were determined:

Urea hydrolyzed (mM)  = (Ammonium (mM) - 820.78) / 4.2962 (in the case of pure urea, Sigma grade)

= (Ammonium (mM) – 593.07) / 4.9711 (in the case of fertilizer urea)

Urea hydrolyzed (mM)  = (Total Ammonia (mM) – 544.38) / 38.064 (in the case of pure urea, Sigma grade)

= (Total Ammonia (mM) – 1152.4) / 37.42 (in the case of fertilizer urea)

The urease activity can be calculated using the linear relationship between the rate of the ammonium (mM.min$^{-1}$) or total ammonia (mM.min$^{-1}$) formation, and the rate of urea hydrolysis (mM urea hydrolysed.min$^{-1}$).

Correspondence between the NH$_4^+$ readings by the sensor and the values of NH$_3$-N obtained using the Nessler method at the completely hydrolysis of various concentration of two urea types
Figure B1.3. Correlation between the two types of measurements (Nessler method and the selective ion sensor measurement), using urease type IX (Sigma) for completely hydrolyzing the pure urea (a) or fertilizer urea (b) at different concentrations 80 mM, 160 mM, 330 mM, 500 mM.

B2 - Conversion of conductivity to urea hydrolysed

A standard curve of the conductivity values resulting from complete hydrolysis of several concentration of urea (Sigma urea and fertilizer urea) by purified urease (Urease Type IX from *Canavaliaensiformis*) purchased from Sigma, at 30°C, was generated.
From these figure, the following relationship were determined:

\[
\text{Urea hydrolyzed (mM)} = \frac{\text{Conductivity (mS)}}{0.1} (\text{in the case of pure urea})
\]

\[
= \frac{\text{Conductivity (mS)}}{0.12} (\text{in the case of fertilizer urea})
\]

The urease activity can be calculated using the linear relationship between the rate of conductivity (mS*min\(^{-1}\)) changes and the rate of urea hydrolysis (mM urea hydrolysed*min\(^{-1}\)).

The Specific Urease Activity (Urease Turnover Frequency or specific EA) was defined as the amount of urease activity per unit biomass and calculated according to the following equation:

\[
\text{Specific Urease Activity} = \frac{\text{Urease Activity (mM urea hydrolysed min}^{-1})}{\text{Biomass (OD\text{560})}}
\]
APPENDIX C

Biomass evaluation

The evolution of the Biomass has been evaluated using different methodologies in small samples taken at different time intervals from the cultural medium sown with *S. pasteurii*:

- **Optical Density** was measured at 600 nm, using a Beckman DU 640 Spectrophotometer. The measurement unit is rau (relative absorbance units).

- **ATP** was measured using a NG Luminometer (3M Health Care) and surface ATP probes Clean-Trace™ (20 µL of sample). The measurement unit is RLU/mL (Relative Light Units/millilitre). The ATP determination is linked with the viable cell activity.

- **CFU** (Colony Forming Units). Serial dilutions were made using sterilized deionized water and small aliquots (100 µL) were plated on the Petri dishes with solid nutrient medium (30 g/L CASO, 20 g/L urea, Agar 15 g/L). The measurement unit is the number of colonies forming unit/millilitre of sown sample and counted after 48h of incubation at 30°C.

The correlation within the various parameters that could be measured for evaluating the biomass is showed in Figure C1 where is possible to evidence the complete correspondence among the different methodologies.
The calcite precipitation experiment was also conducted to investigate the suitability of potential nutrient substitutes and to investigate the dependence on cultivation temperature (D3.32 and [Error! No se encuentra el origen de la referencia]).

Fig C1 - Relationship between the different parameters used for quantifying the biomass: ATP, CFU and OD600.