Chapter

Experimental Investigation of Biomass Attachment to Wastewater Reactors

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Abstract

Attached mass bioreactors have extensively been adopted in the last decades when specific needs have suggested this choice. Benefits and advantages of this multi-faceted technology in wastewater treatment processing are well known, along with the kinetic and mass transfer aspects regarding their operation, essentially belonging to the mass transfer with chemical reaction theory applied to enzymatic catalysis, referred to as Languimur-Hinshelwood kinetics, notably Monod/ Michaelis Menten equations. On the other hand, a consolidated literature has dealt with many aspects of the development of strain colonies forming a biofilm. However, a few works have been devoted to the systematic analysis of its physiology, within the framework of the wastewater management of complex substrates and high-loads effluents. This article presents the experimental findings of a research activity covering the junction area between microbiology and bioreactor engineering, against a multifaceted set of operating parameters directly affecting health and stability of the attached biomass. In this respect, important results have been obtained, providing guidance on the attached mass reactor start-up, steady- state operation, impact of xenobiotic substrates, role of nutrients, filaments and foam formation, as well as qualitative aspects of the post-treatment effluent.

Keywords: attached mass, biofilm, wastewater, nutrients, adhesion test

1. Introduction

The adoption of attached mass reactors for the biological treatment of wastewater started in the late 1800s early 1900s [1]. Trickling filters have been used much earlier than the fundamentals of biochemical engineering applied to sewage purification had been established. During the early 1950's, development of plastic media resulted in the introduction of innovative packing and during the early 1960s rotating discs, included in several chemical process, have begun to be considered for oxygen transfer [1]. In the last 40 years submerged attached growth aerobic processes became established options for wastewater treatment, taking the advantage of a reduced space required with respect to activated sludge [2]. Nowadays, the combination of attached growth/activated sludge systems, also referred to as hybrid process or Integrated Fixed-Film Activated Sludge (IFAS) is an optimized alternative, as well as the Moving Bed Biofilm Reactor (MBBR), which typically ensure an increased treatment capacity, reduced sludge production and handling, low spaces and independency of a secondary clarifier. All attached mass systems have to take into account that mass

transfer is the rate-limiting-steps of the overall biodegradation process. In addition, biofilm quality, structural properties and stability greatly affect the overall process performance, as well as effluent quality and conditions for foam formation. Consequently, the characterization and the understanding of physiology of the biofilm is an essential key for a correct management of the biodegradative process.

2. Background

Historically, Van Leeuwenhoek is credited as the first scientist who observed microbial biofilm formed on a surface [3]. Important contributions to the understanding of the mechanisms and circumstances according to which adhesion takes place were taken by Characklis [4] and Costerton et al. [5]. In the last decades, kinetic and mass transfer aspects of attached biomass have been well studied and parameterised in the scientific and technologic literature [2, 6]. Diaco and Eramo [7, 8] carried out one of the most complete studies combining diffusional and kinetic aspects with the structure of the attached mass. Oxygen transfer from the bulk liquid to the bacterial colony surface and subsequent kinetics follows the general laws of absorption with chemical reaction on a surface layer, as described in the early works of Sherwood [9] and Astarita [10]. On the other hand, kinetics taking place in a biofilm has extensively been studied according to Michaelis- Menten-Monod scenario and successfully framed within Langmuir-Hinshelwood and Hougen - Watson reaction schemes, according to the formalism of heterogeneous catalysis [11]. Williamson and McCarty [12] provided one of the first approach specifically related to the biofilm, showing the correlation between Fick diffusional phenomena and Monod kinetic theory. Authors such as O′ Toole et al. [13] and many others have studied the biofilm structure with the aim to identify how diverse was the mass microbiological behavior with respect to the suspended structure, as to the metabolic, pathogenic and clinical scenarios. More recently, several authors, such as Muslu [14] and Feng et al. [15] have worked on modeling further biofilm as a chemical reaction site, bringing specific contributions to the definition of the biodegradation rate and of the related conditional factors, within the theoretical frame built up by early authors such as Atkinson and Davies [16] and La Motta [17]. Naz et al. [18] evaluated the biofilm succession on stone media and compared the biochemical changes of sludge in attached and suspended biological reactors operated under aerobic and anaerobic conditions. Ercan et al. [19] have studied the biofilm development conditions on different surfaces, predominantly from the point of view of the relationship between the structural and geometrical features of the supports and the process variables, such as oxygen transfer and chemical reaction rate for production purposes. As a matter of fact, a large and consolidated literature has been produced about the characterization of biofilm and attachment mechanism, predominantly either analyzing the process engineering aspects of the bioreactor schemes or studying the purely microbiological and clinical topics per se. Relatively few efforts have been devoted to the analysis of the physiology of the attached biomass, notably dealing with the effects of nutrients, of substrate origin, of its transient behavior in terms of formation rate and stabilization framed within the biodegradation of complex pollutants contained in the wastewaters.

3. Unknown variables and uncertainties

Jenkinson and Lappin-Scott [20] define biofilm as *the microorganism consortium which develops at the interface solid–liquid or liquid–gas*. Gottenbos et al. [21] have

pointed out that *the biofilm is a specific micro-ecosystem inside which different microbial strains effectively cooperate to get protection from ambient stress and to promote nutrients absorption*.

Formation and development of biofilm is assumed to take place according to the following phases, [22–24]:

1. *Adhesion of some planktonic microorganisms to a surface*

2. *Consolidation of a preliminary film layer promoted by Extracellular Polymeric Substances (EPSs) via hydrogen-type bond*

3. *Formation of a monolayer by bacterial strains grown on it*

4. *Development of a three-dimensional biofilm, consisting of a composite structure made of additional planktonic microorganisms and inert materials-EPSs (75 – 95%), cells (5 – 25%)*

5. *Dispersion and expansion of the biofilm*

All these phases are mediated by and are related to specific metabolic factors, which have a specific role in the progression of the process. The definition and characterization of the related parameters are of paramount importance to establish the conditions for a biofilm to be formed and stably persist in an aqueous medium. Parameters and factors affecting biofilm formation, development and effectiveness are very numerous, ranging from metabolic chemistry to genetics, hydrodynamics and transport properties. In the experimental research programme underpinning the present article, a specific focus has been made on those which were expected to have a direct, significant and macroscopic effect on the effectiveness of the wastewater treatment process.

3.1 Nutrients and thermochemical environment

Nutrients, temperature and pH significantly affect the biofilm formation and its behavior. Specifically, effect of temperature and pH is substantially known, whereas, even if a basic understanding of nutrients role has been achieved, often monitored in terms of C/N and C/P ratios, the assessment of biofilm behavior with time and with regards to changing nutrients respectively is important [25].

3.2 Surface and hydrodynamics

Surface and hydrodynamics play an important role. In order for the attachment process to start, contact of cell with solid surface is required, followed by a rapid bond. Strengthen and rapidity of these bonds are rather known, depending on a series of chemical–physical interactions and biological process which lead to a reversible adhesion [26]. This reversibility is to be considered as purely theoretical, as, if a minimum set of bonds has been achieved, the cumulative effect is sufficient to make the adhesion permanent [27]. Once again, nutrients and gram reactivity as well were expected to play an important role both in terms of adjusting the attachment capability to the specific surface and in terms of the transport properties in the aqueous medium, such as viscosity, which, in turn, belongs to the hydrodynamic part of the system.

3.3 Nitrogen contents and filamentous forms

Formation of filamentous forms is a particular drawback of attached growth systems adopted in wastewater treatment. In this respect an important parameter is a high COD value and, connectedly, nitrogen balance [28]. This aspect deserves a specific attention for the possible effects on the correct development and functional characteristic of the biofilm.

3.4 Toxic substances

This is a crucial parameter in wastewater management and possibly is more critical for attached mass bioreactors [29]. In this respect, an indicative approach could be the determination of the difference of the biofilm structure and functional capability against substrates showing an increasing leaving of toxicity.

4. Objectives and novelty of the research

This article presents the findings of an experimental research, conducted at the *Department of Agronomy, Section of Microbiology* of the University of Naples Federico II, Naples (Italy), consisting of the analysis of attached mass formation, based on both mixed industrial-municipal and of the effect of high tannery wastewaters COD, along with the endogenous strains. The research aimed at:

- Studying the main physiological features of the biofilm in relationship to its capacity to settle down and to reduce the COD load of different effluents with different substrates
- Comparing the behavior and the features of the biological structures against different wastewaters
- Appreciating the effects of different nutrients at various dilutions
- Finding out the qualitative aspects of the treated effluents, also considering foam formation, presence of filamentous forms and degree of wastewater clarification, against different parameters and different effluents
- Analyzing the influence of nitrogen substances
- Carrying out a transient analysis from the biofilm formation through its full development to identify any specific growth factors, including CFU/ml

Due to the high level of parameterization related to the high number of variables, experimentally tested, the research is considered a step forward to the understanding of the biomass attached growth mechanism.

5. Materials and research preparation

5.1 Sampling

The study has been carried out on microbial colonies of sludges collected in sterile bottles from Acerra (Italy) mixed industrial-civil wastewater treatment plant

and from CoDiSo tannery wastewater treatment plant of Solofra (Italy), subject and not subject to a physical–chemical process. More precisely, the overall treatment process consists of a sequence of a biological and of a physical–chemical process. Samples have been taken downward of the primary settlement and transported with a thermally insulated bag. After laboratory water removal, the sludges have been distributed into 1.5 ml sterile polystyrene Eppendorf pipettes and 50 ml Falcon tubes. Both have been centrifuged at 10,000 rpm for microbial cell separation and fed with PCB nutrient broth at 20% glycerol. PCB composition has been included in **Table 1**. Settled cells have been suspended in a vortex and stored at −20°C. Wastewater has been collected as well with 4 sterile bottles, notably 2 liters upstream and 2 liters downstream of the physical–chemical treatment unit, with the aim to investigate the toxic and substrate diversity associated with the different sampling points. One fraction of this wastewater has undergone centrifugation, suspension of microbial cells in PCB nutrient broth at 20% glycerol and stored at −20°C. The remaining fraction has been frozen for further tests.

5.2 Inoculum preparation

The microbial matrix has been processed after growth induction. Each test has been carried out after defrosting up to ambient temperature and inoculation of 150 ml of 1% PCB broth. The latter has been incubated at 28°C for about 12 hours overnight, in a rotary shaker. This culture medium was the inoculum for the experimental campaign.

5.3 Material list

The following main materials have been adopted for the execution of the experimental study:

- Microbial material and cultures, provided by the Acerra Wastewater Treatment Plant (Italy)

- Nutrients and additional substrates, collected from the inventory of the Laboratory of the Institute of Microbiology of the Faculty of Agronomy of the University Federico II, Portici (Italy)

- Chemicals and reactants, microbiological grade, supplied by Sigma-Aldrich

5.4 Microbial growth and biofilm development with a substrate of given composition

Investigations have preliminary been carried out with the aim to identify the best conditions for the film development with respect to various glucidic forms and different nitrogen concentrations.

Yeast extract	5 g
D-glucose	2 g
Distilled water	1000 ml
pH = 7.0 ± 0.2 a 25°C / Strain storage with sterile 20% glycerol at – 20°C.	

Table 1.
PCB (plate count broth) – liter^{-1}.

5.4.1 Influence of glucidic compounds

Inoculum M9 at different glucidic concentrations has been tested (**Table 2**). Notably: 1 liter at 1% glucose and 0.05% glucose respectively, and 1 liter at 1% saccharose and 0.05% saccharose respectively. These substrates have been selected in light of their ease to be metabolized so that fast results were expected to be attained with respect to the work objectives. The four substrates, after sterilization at 5% inoculum, have been put in a 1.5 liter flask equipped with two air-in / air-out capillaries for sterile air blowing. Each flask has been equipped with a 2 cm2 Petri dish, adopted as attachment surfaces. The culture has been conducted under hood for 24 hours at ambient temperature of around 25°C. Suspended bacteria growth rates have been measured during this time, by means of spectrophotometric readings at 600 nm, and utilizing, as reference, the relevant blank substrates. During this phase, visual checks have been made as to the clarification degree and foam formation for all cases, and dissolved oxygen has been measured. After 24 hours, fragments of Petri dishes have been collected, gently washed with deionized water and attached microorganism have been stabilized on the solid support by using a Bowin fixative (**Table 3**). Afterwards, the fragments have been dried under laminar hood for 24 hours and fixative removal has been accomplished by means a 50% ethanol solution, and Crystal violet Hucker's solution has been used for attached cells colouration (**Table 4**). Microscopic observations have allowed for a qualitative-quantitative evaluation of the attached biomass.

5.4.2 Influence of nitrogen-containing compounds

The same procedure adopted for glucidic substances has been followed for M9 nutritive standard substrate, modified with nitrogen - containing compounds. 3 liters of nitrogen-containing mineral substrate (ammonium chloride) have been prepared, notably 1 liter at 0.1%, 1 liter at 0.2% and 1 liter at 0.4%, and 1 liter of organic nitrogen-containing substance (pancreatic digest of casein) at 0.5%. In

$NaHPO_4$	6 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g
D-glucose (20 g/100 ml solution)	10 ml
$MgSO_4 \cdot 7\ H_2O$ (246.5 g/ l solution)	1 ml
Thiamine · HCl (10 mg / 20 ml solution)	1 ml
$CaCl_2$ (14.7 mg/100 ml solution)	1 ml
Deionized water	1000 ml

Table 2.
M9 medium – liter^{-1}.

Saturated solution of picric acid	15 ml
Formalin	5 ml
Glacial acetic acid	1 ml

Table 3.
Bowin fixative.

Solution **A**:	
Crystal – violet	2 g
Ethanol 95°	20 ml
Solution **B**:	
Oxalate ammonium	0.8 g
Deionized water	80 ml

Table 4.
Hucker crystal - violet.

addition to being a fundamental element of the biochemical balance of the microbial cultures, the parametrization of nitrogen content has been considered as a fundamental information to assess the behavior of the biofilm. Also for this inoculum, the microorganism growth rate and the biofilm development has been assessed, in addition to the clarification degree, foam formation and filamentous forms.

5.5 Observation of microbial growth and biofilm development in industrial wastewater

Four microbial cultures have been prepared utilizing tannery wastewater as substrate. Specifically:

- Untreated wastewater (IN)
- Wastewater with chemical–physical treatment (OUT)
- Diluted 1:1 untreated wastewater (IN 1:1)
- Diluted 1:10 untreated wastewater (IN 1:10)

The substrates have been collected in a flask and sterilized at 121°C for 15 minutes, then inoculated with at 5% with a microbial sludge collected from the mixed sludges. Microbial growth has been promoted as described previously, as well as for the procedure for air blowing, fragments collection, evaluation of attachment, clarification and foam formation. The development of microorganism has been checked after 24 hours according to the scalar dilution's method and PCA bacterial count on Petri dish (**Table 5**).

5.6 Isolation and purification

Microorganisms of 3 samples have been isolated and then purified. Specifically:

- Attached biomass of dish fragment of previous test
- Sludges collected from mixed wastewater
- Tannery wastewater treated in the physical–chemical unit

PCA has been utilized as sterile agar culture medium. Isolation has been accomplished through suspension dilutions (up to 10^{-9}) of samples and plating by inclusion of 1 ml for each dilution. After incubation for 24 hours at 27°C, largest colonies have been purified by smear on PCA plating. Purified strains have been stored at

Yeast extract	2.5 g
Pancreatic digest of casein	5 g
Glucose	1 g
Agar	15 g
Deionized water	1000 ml

Table 5.
PCA agar– liter^{-1}.

−20°C in PCB at 20% glycerol and at 4°C in slant test tubes, to be collected for the subsequent identification tests. On the same dishes, for sludges and wastewater, prior to smear purification, a bacterium count of colonies of dilutions showing the greatest growth has been made and the results have been expressed in CFU/ml.

5.7 Identification tests

Three main characteristics have been considered with the aim at systematically framing isolated strains: gram-reaction, catalase and cellular morphology.

5.7.1 Gram-reaction test

Gram reaction has been accomplished according to Gregersen [30], adopting a 3% KOH solution, wherein one colony is dissolved and then the loopful is removed and checked for viscosity. A significant viscosity change is indicative of Gram negativity, whereas an unchanged viscosity reveals the Gram positivity. The rationale is based on Gregersen's observation that Gram+ withstand potassium hydroxide whereas in Gram- cellular wall is destroyed and DNA is released resulting in a viscosity change.

5.7.2 Catalase test

Presence of catalase has been sought through the simple hydrogen peroxide test [31]. Notably, observing the reaction oxygen presence can be detected by formation of bubbles, visible with the naked eye. Catalase test protocol originally proposed by Gagnon [32] has substantially been followed for catalase determination.

5.7.3 Microscopic Observation

Cells shape has been controlled through microscopic observation.

5.8 Test of adhesion

This test has been carried out by the adoption of strain B_1, as it constitutes most part of biomass isolated microorganisms. This test allows one to quantify attached mass through the measurement of the optical density of a mature biofilm on a plastic surface [33]. In this respect, Christensen's method has been applied based on the modifications described by Baldassarri et al. [34]. In this study, 7 dilutions of overnight cultures at 28°C have been made in order to obtain, in addition to an evaluation of the strain attachment characteristics, also a relationship between cellular concentration and bioattachment, at the following dilutions:

- 2.5%
- 5%

- 10%
- 20%
- 40%
- 80%
- Culture broth

5 ml have been withdrawn from each dilution, 1 ml of which has been used to prepare suspension-dilutions with the aim to determine the bacterial load corresponding to each sample. Portions left have undergone spectrophotometric readings (600 nm). All samples, except lowest dilutions, have been centrifuged for 10 minutes at 10,000 rpm and, further to supernatant elimination, cells have been resuspended in sterile broth. 2 ml have been collected from each dilution and distributed into 10 pits (200 μl each) of a 96-wells Enzyme-Linked Immunosorbent Assay (ELISA) plate with flat bottom. The plates, provided with 10 wells filled PCB sterile broth as blank sample, have been incubated at 28°C for 24 hours. The broth has been then gently removed from the wells by means of Pasteur pipettes and wells have been washed three times with sterile deionized water to remove unattached cells. This phase has been conducted very carefully as it often causes removal of attached cells also, resulting in false findings. Unlike proposed by Christensen et al. [33], sterile pipettes have been preferred to direct immersion of plates into clean water, in order to minimize any disturbances and handling. Attached cells have then been fixed by exposure to 60°C for 1 hour and colored by Hucker crystal violet. Plates have been rinsed until rinsing water has been completely colorless, then overturned and dried for 30 minutes at 37°C. Biofilm density has been assessed by a spectrophotometer for ELISA plates, after calibrating the blank sample of the instrument with the wells containing non-inoculated sterile broth. Readings have been taken at 550 nm. According to the adopted protocol, mean of taken readings for each well has been interpreted as:

- Positive: ≥ 0.24
- Weak: ≥ 0.12 and < 0.24
- Negative: < 0.12

5.9 Observation of the growth-curve shown by a pure culture in presence of a complex substrate

A study has been carried out with the aim to investigate the growth of a B_1 strain with a substrate consisting of pure butanol only. In this respect, two tests have been performed:

- Synthetic substrate consisting of 1000 ppm of butanol inoculated with 5% B_1 strain
- ATP substrate inoculated with 5% B_1 strain

The Inoculum has been prepared from a strain stored in a freezer on slant PCA. Sterile broth culture contained by a 20 ml tube has been inoculated with B_1 strain, and incubated for 15 hours overnight at 28°C. The culture obtained was the 10%

Pancreatic digest of casein	12.5 g
Glucose	10 g
Yeast extract	7.5 g
NaCl	5 g
K_2HPO_4	5 g
Sodium citrate	5 g
$MgSO_4 \cdot 7\ H_2O$	0.8 g
$MnCl_2 \cdot 4\ H_2O$	0.14 g
$FeSO_4 \cdot 7\ H_2O$	0.04 g
Tween 80	0.2 g
Na_2CO_3	1.25g
Thiamine · HCl	1 mg
Deionized water	1000 ml
pH = 7.0 ± 0.2 a 25 °C.	

Table 6.
APT broth – liter^{-1}.

inoculum to 200 ml of APT broth (**Table 6**), incubated in a rotary-shaker for 15 hours at 28°C, resulting in the inoculum for the two tests. The two substrates have been kept under chemical hood for 48 hours at ambient temperature. At the same time, a fraction of the following substrates has been stored in sterile flasks:

- Synthetic substrate consisting of 1000 ppm of butanol inoculated with 5% sterile APT

- APT sterile cultural substrate

These substrates have been used as blank samples in the periodical spectrophotometric readings (600 nm) taken for either test.

5.10 C.O.D removal by a pure culture and by a heterogeneous culture (sludge) of tannery wastewater not subject to physical–chemical treatment

Two sterilized samples of untreated tannery wastewater collected in two flasks have been adopted as substrate for B_1 strain. The two samples have been inoculated at 5% with the strain and with sludge respectively. Following exactly the procedure illustrated previously, after 24 hours, 50 ml of substrate have been extracted, transferred into sterile Falcon polystyrene tubes and centrifuged at 10,000 rpm for 10 minutes, to remove microbial cells. Supernatant, collected in sterile medium, has undergone C.O.D test with potassium bichromate 0.0417 M [35]. The protocol utilizes a solution 0.25N of FAS (ferrum(II) ammonium sulphate) as titrant, and C. O.D has been calculated according to the formula:

$$C.O.D. = DF \bullet (V_b - V_s) \bullet M \bullet 800 \qquad (1)$$

where:

- V_b is the average volume (ml) of iron(II) ammonium sulphate solution used in the titration of blank solution

- V_s is the volume (ml) of iron(II) ammonium sulphate solution used in the titration of the samples

- DF is the dilution factor

- M is the molarity of standardized iron(II) ammonium sulphate solution

5.11 C.O.D removal by a pure culture in a pure substrate

The same strain has been tested for the removal of the C.O.D. of two synthetic substrates consisting of 1000 ppm of butanol, inoculated with 5% sterile APT and 5% B_1 respectively. Growth stimulation, post-treatment and determination have been performed as described for previous tests.

6. Results and discussion

6.1 Sampling and storage of microbial material

Microbial materials utilized for the present study have been collected from two wastewater treatment plants, notably:

- Acerra (Italy) industrial and civil wastewater treatment plant

- Solofra (Italy) tannery pole wastewater treatment plant

Acerra wastewater undergoes a traditional activated sludge treatment process, following preliminary removal of gross materials and primary settlement. Activated sludge produced is split between 98% recirculated fraction and 2% fed to thickening and stabilization processes. Colonies populating the sludge flocs have been included in **Table 7**. Samples associated sludge parameters are B.O.D.5 = 20 mg/l and active mass concentration = 2600 mg/l. Solofra plant implements a biological/physical–chemical process over a C.O.D of 7000-8000 mg/l. After a preliminary phase of precipitation with limestone, polyelectrolyte and ferric chloride/aluminum sulphate, wastewater is supplied to a biological plant, with a final activated carbon adsorption stage for the removal or coloring and residual organic matter. Activated sludges of Solofra plant are composed predominantly of proteobacteria, Bacteroidetes, firmicutes with a presence of actinobacteria, planctomycetes and chloroflexi [36].

Aspidisca costata	Lionotus lamella
Colpidium colpodum	*Paramecium caudatum* et petridum
Didinium	Opercularia
Carchesium polypium	*Arcella vulgaris*
Epistylis	*Rotaria rotatoria*
Philodina	Nematodi
Stylonychia mytilus	Crustaceans molds
Vorticella microstoma	
(Courtesy of Acerra wastewater treatment plant)	

Table 7.
Microorganisms of activated sludges.

Inventory of bacterial matters consisted of 1.5 ml 300 Eppendorf, of which 100 of sludge, 100 of untreated water and 100 of physically-chemically treated water. Also, No. 105 50 ml Falcon tubes, of which 35 of sludge, 100 of untreated water and 100 of physically-chemically treated water. Biodegradative inherent capacity of Acerra sludges in a suspended lab scale bioreactor about a specific substrate such as ethyl acetate had preliminarily been tested by the author [37].

6.2 Microbial growth and development of biofilm in sugars-broth

Development of the biofilm is promoted by organic molecules through the formation of a conditioning layer on solid surfaces. M9 has been selected with the aim to prevent false results. Carbon source for this inoculum is glucose only, so that observed attachment could be put in relationship with bacteria capabilities only.

6.2.1 Influence of sugars

Bacteria growth with sugar type and concentration has been obtained through spectrophotometric controls shifted by 1 hour. The findings have been depicted in **Figure 1** [38]. The curves have been obtained by data reparameterization according to Gompertz equation as modified by Zwietering et al. [39]. As expected, growth of microorganisms is characterized by a latency phase, an exponential growth, a steady-state growth and death. Resulting growth with time can be described by the following parameters:

- The maximum growth rate μ_m, coincident with the tangent at the flex point
- The duration of the steady-state phase, λ, that is the intercept of the tangent, on the horizontal axis
- The asymptote A, defined as the maximum attained value:

$$y = A \cdot exp\left\{-exp\left[\frac{(\mu_m \cdot e)}{A} \cdot (\lambda - t) + 1\right]\right\} \quad (2)$$

After 24 hours substrate clarification, foam formation and presence of filamentous forms caused by bacteria growth have been evaluated as shown in **Figure 2**. It

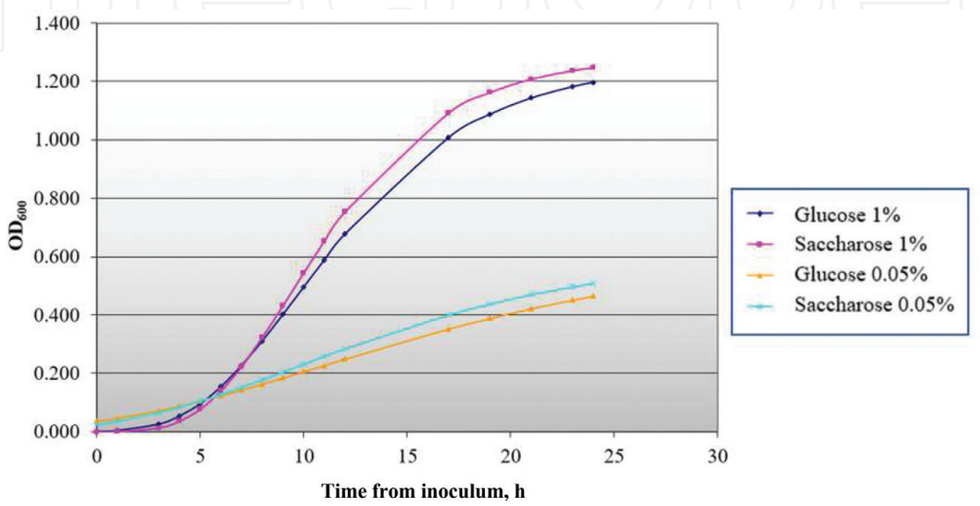

Figure 1.
Microbial growth in sugar-broth.

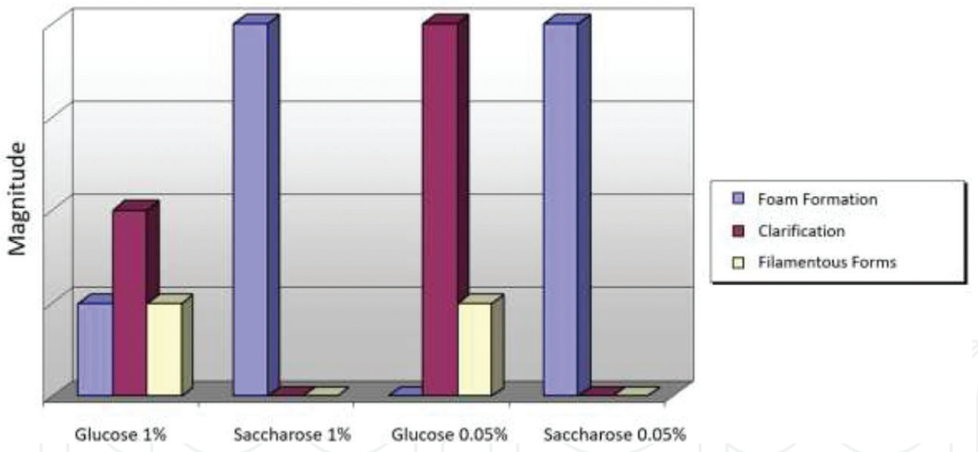

Figure 2.
Effects of substrate of microbial growth in sugar-broth.

can be noted that glucose provides better results than saccharose, which produces both at high and low concentrations, a significant foam formation along with an intense turbidity. Glucose 1% promotes a faster colonies growth than glucose 0.05%: the former can be adopted during the start-up, the latter at the steady-state, where effluent quality matters. The microscopic observation of the attached mass, accomplished on 4 fragments for each test, has revealed the presence of filamentous forms in glucose test, as shown in **Figure 2**, where arbitrary unitless relative values have been assigned. However, this does not affect the quality of the effluent as stated before, as to ideal sugars. The microscopic observation has revealed that the fours tests have shown the same results in terms of biofilm formation, as illustrated in **Figure 3**, based again on relative unitless values. The biofilm was well developed and appeared as a dense, uniform and well-structured cells mass. In some instances, the subsequent cellular layers have been observed.

6.2.2 Influence of nitrogen-substances on microbial growth and development of biofilm

Influence of nitrogen on biofilm development has been assessed with a specific test. **Figure 4**. shows that organic nitrogen promotes cell growth much more than mineral nitrogen, whose different concentration does not significantly affect the

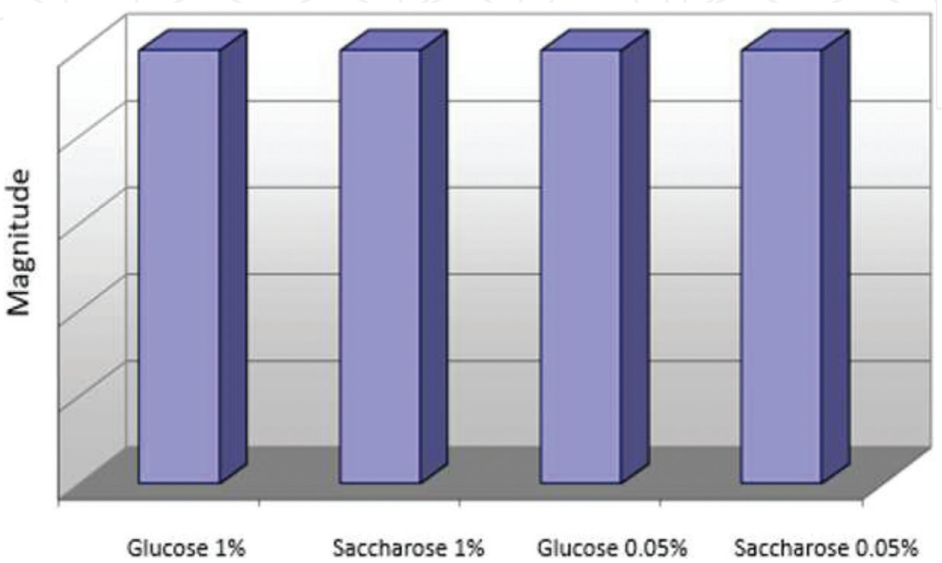

Figure 3.
Biofilm development – Attachment tests in sugar-broth.

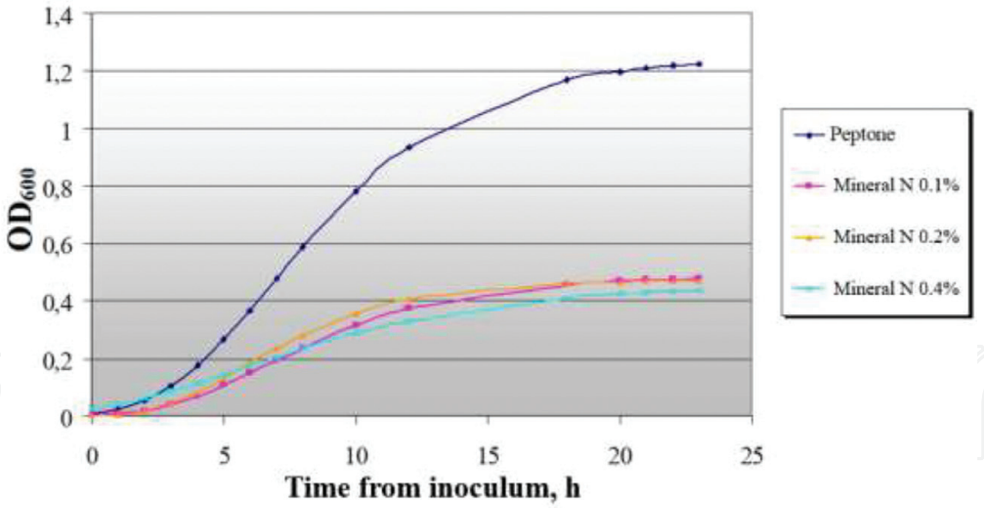

Figure 4.
Microbial growth with nitrogen-compounds.

growth. However, the bigger growth is accompanied by a significant foam formation. This has not been observed when ammonium chloride has been adopted, along with a good clarification, as shown in **Figure 5**. Microscopic observation of the plate fragments collected from the various tests has provided unexpected results (**Figure 6**). Notably, no difference has been detected between biofilms developed with organic and inorganic nitrogen. This disagrees with Mittelman [40] and is in agreement with Abbott et al. [41], according to which the availability of an organic layer next to the attachment surface would be substantially uninfluential, as this would be controlled mainly by electrostatic interactions. Filamentous forms have resulted to be absent in all four tests.

6.3 Microbial growth and development of biofilm in industrial wastewater

Activated sludges collected from Acerra treatment plant have been adopted as microbial matrix, whereas treated and untreated wastewaters coming from Solofra treatment plant (**Table 8**) have been used as substrate. Count of suspended bacteria instead of spectrophotometry was due to the significant TDS and to the related difficulty to calibrate the instrument. Having the count been carried out at irregular intervals during the test, it was not possible to draw a growth curve, but, based on the final bacterial load, it was possible to estimate the growth trend and the

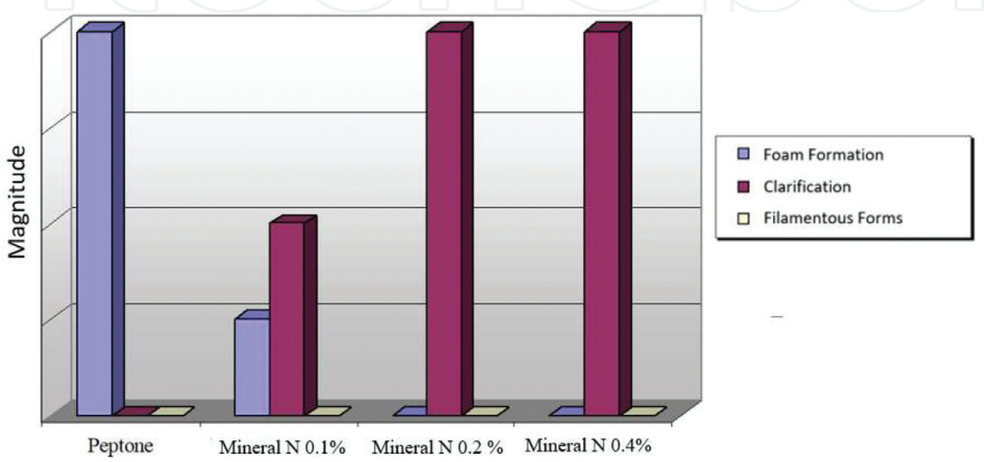

Figure 5.
Effects of substrate of microbial growth with nitrogen-compounds.

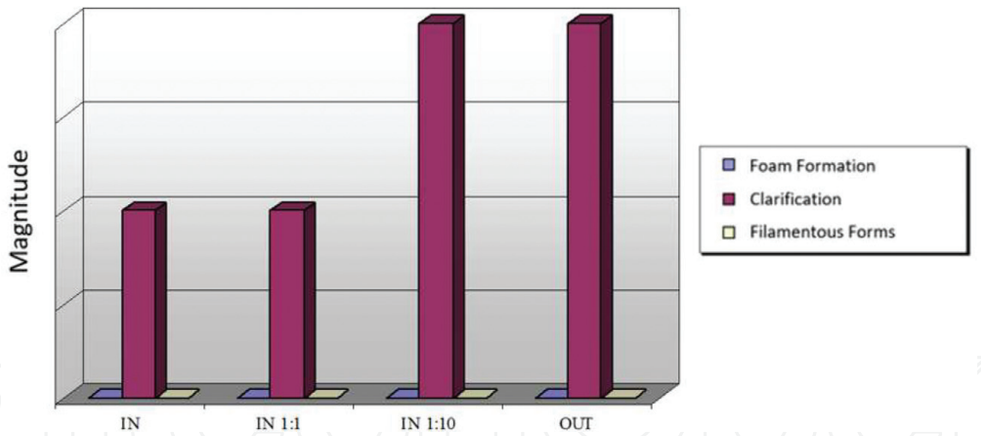

Figure 6.
Tannery wastewater treatment – Quality of the effluent.

COD (mg/l)	8860
TDS (mg/l)	2210
N_4 (mg/l)	100.1
pH	3.6
(Courtesy of Solofra wastewater treatment plant)	

Table 8.
Untreated wastewater characteristics.

survivability of the microbial matrix. Findings of bacterial count are shown in **Table 9**. A longer survivability was observed in wastewaters which underwent chemical settlement and flocculation, as expected from the very high toxic load of tannery effluents. Quality of the effluent in terms of foam formation, clarification and presence of filaments has been shown in **Figure 6** and **Table 10**. Once again, presence or removal of toxic substances are a key driver for the lower or higher degree of clarification. Contrary to the findings of the other tests, which showed same biofilm formation and features, significant even microscopic differences have been identified in this test, as to the attached biomass in the various substrates. A qualitative relative indication of the biomass formation has been shown in **Figure** 7. The layer observed at the microscope even at high toxic concentrations can be

	IN	**IN 1:1**	**In 1:10**	**OUT**
CFU/ml	2E-04	2E-05	4E-05	4E-07

Table 9.
Tannery wastewater. Bacterial count after 24 hours at different dilutions.

	IN	**IN 1:1**	**In 1:10**	**OUT**
Foam formation	Absent	Absent	Absent	Absent
Clarification	Medium	Medium	High	High
Filamentous forms	Absent	Absent	Absent	Absent

Table 10.
Quality of treatment of tannery wastewater.

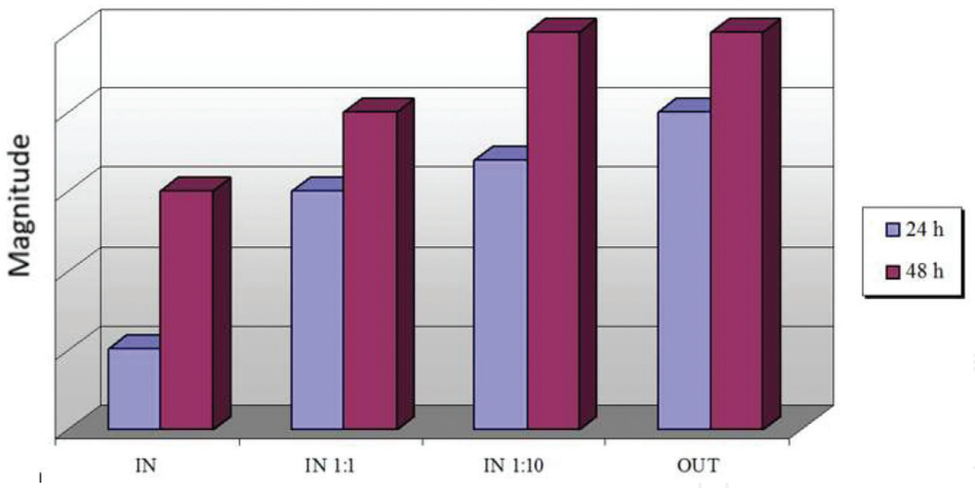

Figure 7.
Biofilm formation in tannery wastewater.

justified by the survival at these concentrations of morphologically different bacterial colonies.

6.4 Isolation and purification

Nineteen strains have been selected from the available samples, notably:

- 5 collected from the attached biomass, indicated with initial B
- 5 collected from the mixed-wastewater treatment plant, indicated with initial S
- 9 from the physically-chemically treatment tannery wastewater, indicated with initial T

It is worth noting that more numerous strains have been taken and untreated tannery effluents have been excluded from the test. Morphology, Gram reaction and catalase test findings have been included in **Table 11**. Morphologically different strains have shown same behavior, and rod shape and $Gram^+$ have been predominant. Bacterial counts executed on mixed wastewater sludges, as well as on treated and untreated tannery effluents, have resulted in 2·108, 2·108 and 4·17 CFU/ml respectively.

6.5 Attachment test

This test has consisted of the determination of the relationship between OD600 values and concentration of cells materials per ml of assumed dilution. **Figures 8** and **9** show the relevant spectrophotometric findings. **Table 12** shows the results of the OD_{550} spectrophotometric findings of the observations on ELISA plates adopted for the attachment test. **Table 12** also shows means and standard deviations related to the 10 times repeated tests for each dilution. The test has revealed an excellent attachment capability of the studied strain.

6.6 Statistical analysis

Experimental results have undergone statistical analysis, with the aim to assess the significance of the differences observed between the analyzed samples, notably,

Strain	Morphology	Gram reaction	Catalase
S_1	Cocci	—	+
S_2	Cocci, diplococci	—	+
S_3	Rods	+	+
S_4	Rods	+	—
S_5	Cocci	—	+
B_1	Rods arranged in chains	+	+
B_2	Cocci arranged in chains	+	+
B_3	Cocci arranged in chains	+	—
B_4	Streptobacteria	+	+
B_5	Cocci	—	_
T_1	Rods arranged in chains	+	+
T_2	Rods arranged in chains	+	+
T_3	Rods arranged in chains	+	+
T4	Rods arranged in chains	+	+
T5	Rods arranged in chains	+	+
T6	Rods arranged in chains	+	+
T7	Rods arranged in chains	+	+
T8	Rods arranged in chains	+	+
T9	Cocci	+	—

Table 11.
Differential characteristics of isolated strains.

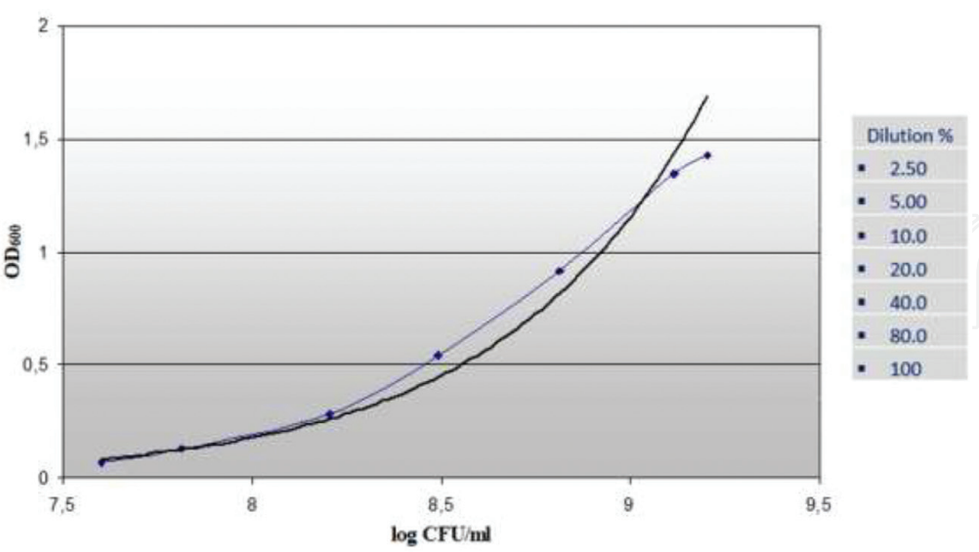

Figure 8.
OD_{600} vs. CFU/ml at different dilution ratios.

through Fischer and Yates variance ratio (F). Based on this ratio, significance of variability due to identified variation sources has been assessed, in relationship with the variability due to error. Here a F has been found to be equal to 380.764, so much greater than the tabulated value (3.14). The analysis has been refined further through Duncan's multiple range test [42]. This was necessary as the means were

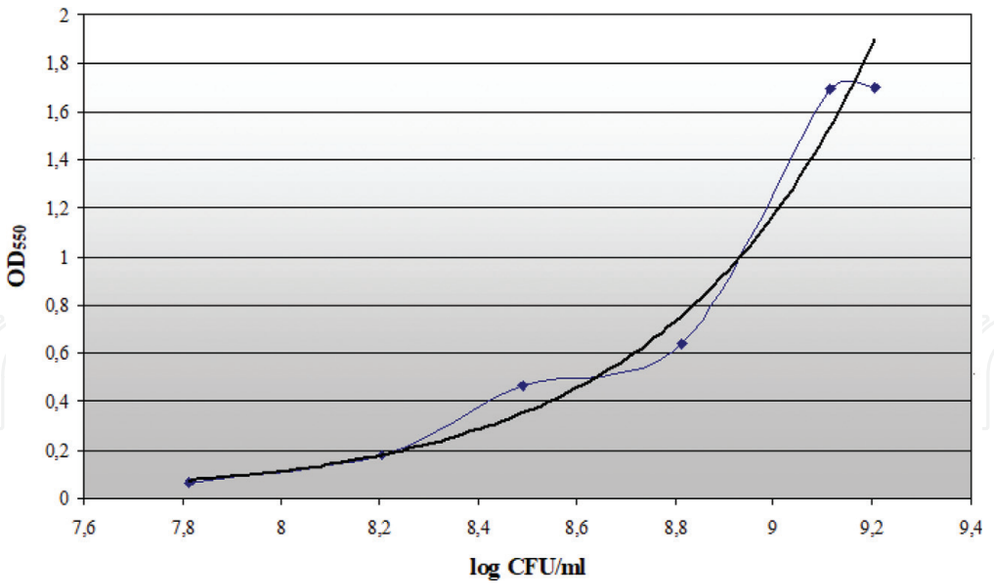

Figure 9.
Attachment behavior vs. CFU/ml.

	A	B	C	D	E	F	Blank
OD_{600}	0.068	0.395	0.505	0.227	1.892	1.763	0.075
OD_{600}	0.141	0.368	0.432	0.859	1.823	1.632	0.085
OD_{600}	0.090	0.495	0.565	0.611	1.632	1.721	0.079
OD_{600}	0.076	0.143	0.357	0.891	1.702	1.963	0.082
OD_{600}	0.134	0.153	0.589	0.695	1.712	1.812	0.060
OD_{600}	0.140	0.231	0.508	0.771	1.638	1.601	0.067
OD_{600}	0.169	0.175	0.571	0.801	1.856	1.897	0.068
OD_{600}	0.129	0.216	0.851	0.685	1.685	1.851	0.074
OD_{600}	0.185	0.220	0.262	0.797	1.886	1.612	0.059
OD_{600}	0.227	0.152	0.362	0.763	1.824	1.843	0.066
OD_{600}	0.135	0.251	0.536	0.710	1.765	1.769	0.0715
Mean	**0.049**	**0.114**	**0.144**	**0.188**	**0.101**	**0.125**	**0.0089**
St.Dev	**0.068**	**0.395**	**0.505**	**0.227**	**1.892**	**1.763**	**0.0750**

A = $6.5 \cdot 10^7$ CFU/ml; B = $1.6 \cdot 10^8$ CFU/ml; C = $3.1 \cdot 10^8$ CFU/ml; D = $6.5 \cdot 10^8$ CFU/ml; E = $1.3 \cdot 10^9$ CFU/ml; F = $1.6 \cdot 10^9$ CFU/ml.

Table 12.
Spectrophotometric check (OD550) on 10-times repeated test – Elisa plate.

different and, in fact, significant differences between the means of the readings of the various cells populations have been found. Specifically, internal variability of the series related to the blank and to the cell concentration of order of magnitude of 107 CFU/ml have resulted to be too high to conclude that means are different. This allows one to state that attachment phenomenon is a direct function of CFU/ml only, within a certain range of cell concentrations: in fact, the growth is weak and independent of the microbial population until around 10^7 CFU/ml, whereas within 10^8 e 10^9 CFU/ml it becomes exponential, after which becomes stable. The results of the statistical analysis have been reported in **Tables 13** and **14**.

	Squares sum	DF	Squares mean	F	Sign.
Between groups	32.030	6	5.338	380.764	0.000
Within groups	0.833	63	$1.402 \cdot 10^{2}$		
Total	32.913	69			

Table 13.
Results of ANOVA univariate.

Reiterations		Subgroups for alfa = 0.05 Means for homogeneous subgroups shown				
		1	2	3	4	5
Blank	10	$7.15 \cdot 10^{2}$				
A	10	0.1359				
B	10		0.2512			
C	10			0.5366		
D	10				0.7100	
E	10					1.7650
F	10					1.7650
Sig.		0.228	1.000	1.000	1.000	0.993

Table 14.
Comparison between means based on variance (Duncan test).

6.7 Growth-curve of a pure culture in presence of a complex substrate

Strain B_1 has been investigated as to its capacity to grow in presence of carbonaceous rich substrate such as ATP and of 1000 ppm of pure butanol in deionized water. For the purpose of the research, complex substrate is meant any substances whose degradation time is longer than those corresponding to municipal wastewater. B_1 shows a rapid growth with ATP reaching the steady state in less than 8 hours, whereas with butanol it is reached in about 3 hours (**Figure 10**). Lack of nutrients

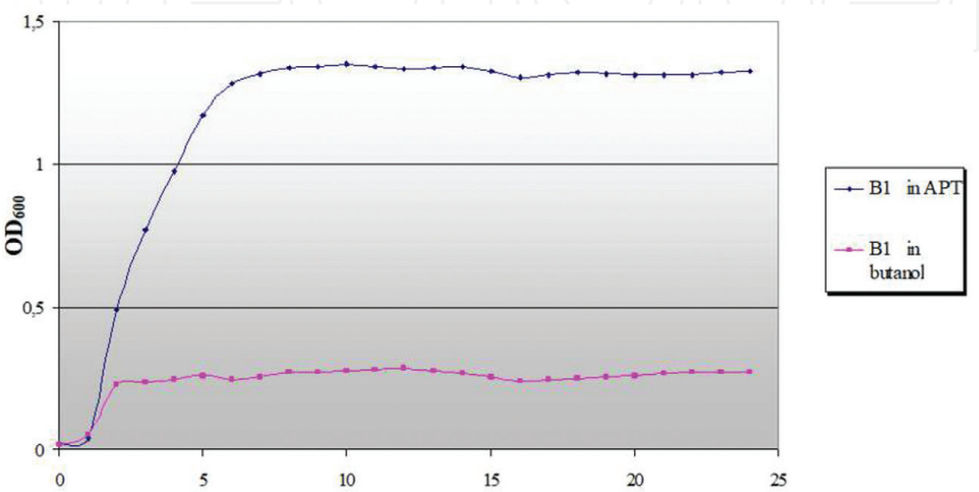

Figure 10.
Spectrophotometric checks for B_1 growth (readings at 600 nm).

plays a specific role in the observed behavior. On the other hand, the exponential growth shows that B_1 tolerates butanol quite well and uses ii for its own development. This confirms Eckenfelder [43] and Benintendi [37] as to the time taken to completely assimilate the organic matter. It can be concluded that the biofilm can be effective in removing COD even in presence of xenobiotic substances.

7. Findings highlights

The Strain B_1 was identified by Marino and Benintendi [44] and Benintendi [37] as *Bacillus alvei*. Based on the evidence described, the following conclusions can be drawn on the attachment mechanism:

- Strain B_1 has shown excellent attachment features according to the parameters indicated by Baldassari et al. [34]. Notably, in correspondence of cell concentration assumed by Baldassarri's protocol (overnight 1:2 culture dilution) a OD_{550} of 0.53 is attained, significantly greater than the minimum value required to defined the strain as positive (OD_{550} = 0.24)

- Attachment depends on a specific cell range, i.e. 10^8 - 10^9 CFU/ml

- Implementing the attachment based on these characteristics could be effective by adopting the strain as inoculum in an attached mass equipment and, complying with the strain needs, the attachment starting phase would be easily promoted.

- Further investigation is required to understand how xenobiotic substances affect strain growth and biodegradative capabilities

8. Conclusions

The experimental research has shown important data related to the knowledge of the physiology of attached mass bioreactors for treatment of complex wastewater.

- Attachment capabilities of bacterial strains have been observed within a broad range of cultural conditions.

- The variation of nutritional conditions results in a selection of microorganisms which, in turn, affects the biofilm structure and thus the quality of the treated effluent, as to clarification and foam formation.

- Contents of mineral nitrogen, instead of organic nitrogen, and glucose instead of saccharose, promote the achievement of a good quality effluent. Specifically, as to saccharose adoption, high concentrations would be required for the start-up, whereas lower amounts should be adopted in the following phases, in order for a good quality effluent to be obtained.

- The presence of toxic substances, even if does not influence significantly qualitative characteristics of the effluent, results in a selective promotion of the microorganisms, which delays the biofilm formation.

- Strains identified as B_1 has undergone specific tests, showing very satisfactory results. This suggests its adoption for the inoculum during the start-up of an attached-mass wastewater treatment plant.

Further studies are recommended to complete the analysis to fully understand the effect of xenobiotic substances on the strain growth, which have been shown to be expectedly well biodegraded, the biofilm formation and stability and the qualitative aspects of the effluent.

The present study offers a good and comprehensive basis for further research phases.

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