Gordonia sp. BS29 as a producer of bioemulsifiers: physiological and molecular characterization

Manuela Pintus

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.......to my father and to my beloved friend Alessandro.....

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Chapter 1

Introduction

1.1 Summary of the Thesis

Polymers from renewable resources have world-wide interest and are attracting an increasing amount of attention for predominantly two major reasons: environmental concerns and the fact that petroleum resources are finite. Generally, polymers from renewable resources can be classified into three groups: natural polymers such as starch, protein and cellulose, synthetic polymers from bioderived monomers (e.g. polylactic acid), and polymers from microbial fermentation (e.g. polyhydroxybutyrate) (Yu, 2009 [42]).

Many polymers of biological origin (biopolymers) have the same activities of their synthetic counterparts but they have higher biodegradability, lower toxicity, activity under a wider variety of condi-

tions. Moreover, they can be produced from cheaper and renewable substrates (Banat et al., 2000 [5]). Nowadays, several biopolymers are produced by microbial fermentation. Among them, microbial extracellular polysaccharides, also named exopolysaccharides (EPS), are water soluble polymers secreted by microorganisms during fermentation. They have found a wide range of applications in the food, pharmaceutical and other industries due to their unique structures and physical properties. Some of these applications include their use as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubrificants, film formers, thickening, and suspending agents (Paul, 1986 [29]).

Currently, polysaccharides obtained from plants (e.g. guar gum, arabic gum, or pectins), algae (e.g. alginate, carrageenan or agar) and crustacean (e.g. chitin) still dominate the market of these commercial products with microbial polysaccharides (e.g. xanthan gum, gellan, pullulan and bacterial alginate) representing only a small fraction due to their higher production cost (Canilha et al., 2005 [7]). Nevertheless, the increased demand for natural polymers for various industrial applications has led in recent years to a renewed interest in exopolysaccharide production by microorganisms (Kumar et al., 2007 [21]). Microbial EPS offer i) consistency resulting from freedom from geographical and/or seasonal variation in production composition, ii) purity and iii) controlled production at high rates and yields, leading to tailored products (Morin, 1998 [27]).

For all these reasons, EPS have been of topical research interest. Newer approaches are carried out today to replace the traditionally used plant gums by their bacterial counterparts. These biopolymers are rapidly emerging as industrially important and are gradually becoming economically competitive with natural gums produced from marine algae and other plants also thanks to more economically competitive production processes developed by novel strategies (Kumar et al., 2007 [21]); Bajaj et al., 2007 [4]).

Microorganisms producing EPS have been identified in various ecological niches and they are found in various species of bacteria, algae and fungi. Environments offering a high carbon/nitrogen source ratio are susceptible to be enriched in polysaccharide-producing microorganisms. Carbohydrate-rich environments are the effluents from the sugar, paper or food industries as well as wastewater plants and sewage sludge. Moreover microorganisms utilizing petroleum derivatives might be a source of EPS-producing strains as some of them produce bioemulsifiers (Morin, 1998 [27]). Among the EPS produced by oil degrading microorganisms, the best studied are bioemulsifiers from bacterial strains belonging to the *Acinetobacter* genus.

To date, the only bioemulsifier produced on industrial scale and available in market is *emulsan*, a polyionic lipopolysaccharide produced by *Acinetobacter lwoffii* RAG-1. It is marketed by Petroleum Fermentation (Netherlands) for use in cleaning of oil - contami-

nated vessels, oil spill remediation, microbial enhanced oil recovery (MEOR), and in facilitating the pipeline transportation of heavy crude oil.

Emulsan synthesis in *Acinetobacter lwoffii* RAG-1 represents the only system for which not only the chemical structure, property and applicability have been fully elucidated but the biosynthetic pathway has also been completely identified and a physiological role hypothesized. The industrial use of the other bioemulsifiers produced by oil degrading bacteria, like the use of microbial EPS in general, is limited due their high production cost compared to chemical emulsifiers. The main reasons for this include poor strain productivity, necessity to use expensive substrates, high production cost and inefficient downstream processing technologies. Thus, the ability to manipulate the metabolism of the producing strain to use cheaper substrates and the improvement of the process technology to facilitate product recovery may increase the economical competitiveness of microbial emulsifiers (Shete et al., 2006 [32]).

The goal of this work was to characterise the production of bioemulsifiers by the strain *Gordonia* sp. BS29, an hydrocarbon - degrading bacterium isolated from a diesel contaminated soil, in order to develop a cost-efficient process for their production.

Gordonia has been recognised as an emerging genus in biotechnology. Several strains were recently patented in industrial and environmental applications. The number of publications describ-

ing novel compounds produced by *Gordonia* strains has increased steadily. *Gordonia* species produce a variety of compounds which are useful for various applications, such as L-lysine, carotenoids and the extracellular polysaccharide gordonan (Arensköttern et al., 2004 [2]).

Gordonia sp. BS29 is able to produce and release lipopolysaccharidic emulsifying agents when grown both on water-soluble and insoluble substrates (Franzetti et al., 2007 [14]). Microcosm experiments have demonstrated that the BS29 bioemulsifier is a promising washing agent for the remediation of hydrocarbon-contaminated soils (Franzetti et al., 2009 [13]). For all these reasons, *Gordonia* sp. BS29 can be considered as a suitable microorganism to be employed for the design and development of a bioemulsifier production process.

However, as with regard to biopolymer production, the major bottleneck for an industrial application of bioemulsifiers is the production cost. Different strategies have been adopted to make biotechnological processes cost-competitive: i) use of cheap substrates or wastes; ii) optimisation of fermentative conditions and recovery processes, iii) selection of over-producing strains (Mukherjee et al., 2006 [28]).

In order to evaluate the feasibility of cost-efficient process, a variety of cheap and renewable and waste substrates was tested for the production of emulsifiers by the strain BS29. Molasses from

sugar-beets, plant-derived oils and oil wastes from food industry (such as waste frying oil) were effective and cheap substrates for bacterial growth and bioemulsifier production. Experimental design techniques were then employed to optimise the culture medium composition for the bioemulsifier biosynthesis by BS29 strain. For this purpose, a spectrophotometric assay was developed in order to quantify the variation in bioemulsifier production and release in cultures. Results of this analysis indicated that the type and concentration of the carbon source and the concentrations of buffer phosphate and magnesium significantly affect the emulsifier production. In order to scale up the system, preliminary experiments were also carried out in a 3-liter mechanically stirred fermentor. All these experiments were performed in *batch* mode.

Overall data showed that synthesis and release of the bioemulsifier by the strain BS29 occur during the exponential phase of bacterial growth. Thus, the bioemulsifier produced by *Gordonia* sp. BS29 can be classified as a primary metabolite.

Colony morphology of the *Gordonia* sp. BS29 is smooth when the strain grows on agar media. However, smooth colonies frequently gave rise to rough ones and this phenotypic alteration seems to be irreversible. Moreover, the rough variant is impaired the bioemulsifier synthesis. The smooth-to-rough conversion of the colony morphology has been described in others members of the *Corynebacterineae* suborder and it is associated with the loss of the ability to synthesise

amphiphilic compounds (Moorman et al., 1997 [26]; Iwabuchi et al., 2000 [17]). Based on this premise, the physiological and molecular characterisation of the *Gordonia* sp. BS29 and its morphological variant represents a fundamental activity for the development of the bioemulsifier production process and the elucidation of its biosynthetic pathway.

The analysis of bacterial growth clearly demonstrate that the models describing the kinetics of the two morphotypes were the same on different water soluble carbon sources. Fingerprinting molecular techniques (REP-PCR and AFLP) were used to characterise *Gordonia* sp. BS29 and its rough variant in order to verify if this phenotypic variation has a genetic basis. In order to investigate if the smooth-to-rough conversion is due to rearrangements or complete loss of bacterial plasmid in a rough variant, the plasmid presence of the two morphotypes were also investigated. Actually, the molecular analyses did not highlight genetic differences between the bioemulsifier-producing strain and its non-producing morphological variant.

The research presented in this PhD Thesis was carried out in collaboration between the Department of Evolutionary Biology "Leo Pardi" of the University of Florence and the Department of Biomedical Science and Technology of the University of Cagliari.

Chapter 2

Development of cost-efficient bioemulsifier production process by *Gordonia* sp. BS29

2.1 Introduction

2.1.1 Bacterial exopolysaccharides (EPS)

Varieties of microbial polysaccharides, serving different functions in a microbial cell, may be distinguished into three main types: i) intracellular polysaccharides that provide a mechanism for storing carbon or energy; ii) structural polysaccharides that are components of the

cell wall structures, such as lipopolysaccharides and teichoic acids; and iii) extracellular polysaccharides referred to as exopolysaccharides (EPS) (Kumar et al., 2007 [21]).

Microbial exopolysaccharides are water-soluble polymers being ionic or non-ionic. The repeating units of these exopolysaccharides are regular, branched or unbranched, and are connected by glycosidic linkages (Bajaj et al., 2007 [4]). Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30 kDa) and have a heteropolymeric composition (Kumar et al., 2007 [21]).

A large numbers of microbes produce exocellular polymeric substances composed of polysaccharides, protein, lipopolysaccharides, or complex mixtures of these compounds. The extracellular polymers produced by a variety of microorganisms are largely composed of polysaccharides (95%) with the balance made by lipoproteins or proteins. The polysaccharides are constituted of sugars, uronic acids, sulphonated sugars or ketal-linked pyruvate groups (Sutherland, 1999 [36]). The sugars may be pentoses (D-arabinose, ribose and xylose), hexoses (D-glucose, mannose, galactose, allose, rhamnose and fucose) and amino sugars (D-glucosamine and galactosamine), while the uronic acids include D-glucuronic acid or D-galacturonic acid. The non-sugar components of EPS include acetate, succinate, pyruvate, phosphate and sulphate (Kumar and Mody, 2009 [22]).

The bacterial exopolysaccharides represent a wide range of chem-

ical structures, they are produced by several microbial species and they have multitudinous applications. Bacterial EPS occur in two basic forms: i) as a capsule (e.g. capsular polysaccharide, CPS, K-antigens) where the polysaccharide is ultimately associated with the cell surface and may be covalently bound; and ii) as a polysaccharidic slime which is loosely bound to the cell surface (Kumar et al., 2007 [21]). The increased demand for natural polymers for various industrial applications has led in recent years to a renewed interest in exopolysaccharide production by microorganisms. Bacterial polymers have emerged as new, industrially important, polymeric materials which are gradually proving economically at par with natural gums produced by marine algae and plants. Various exopolysaccharides produced by bacteria have novel and unique physical characteristics and are generally referred to water-soluble gums (Morin, 1998 [27]). Several microbial polysaccharides are now widely accepted products of biotechnology, while others are in various stages of development (Sutherland, 1998 [35]).

Categorizing exopolysaccharide

Microbial exopolysaccharides are classified on the charge they possess. Anionic EPS (possess acidic groups such as carboxyl, phosphate or sulphate) include xanthan, phosphomannan and alginate; neutral EPS include levan, scleroglucan, pullulan, dextran and curdlan and cationic EPS have some free amino groups. Pyruvate ketals

contribute to the cationic nature of exopolysaccharide. They can be further classified based on the type of monosaccharide units they contain. Polysaccharides containing one kind of monosaccharide are called homopolysaccharides whereas those containing different types of monosaccharides are known as heteropolysaccharides. EPS are heterogeneous often species or even strain-specific and can show enormous variability (Kumar and Mody, 2009 [22]).

Physiological role

The precise role of the exopolysaccharide in EPS-producing bacteria, is dependent on the evident in different ecological niches of the microorganism (Kumar et al., 2007[21]). In the natural environments in which microorganisms are found, such polymers may either be associated with virulence, as in the case of plant or animal pathogens, plant-microbe interactions or protection of the microbial cell against desiccation or attack by the bacteriophages and protozoa. In both natural and man-made environments the EPS play a major structural role in "biofilms", the normal form in which the prokaryotic and eukaryotic microorganisms grow while attached to solid-liquid interfaces (Sutherland,1998 [35]). The presence of a gelled polysaccharide around the cell may also have a paramount effect on the diffusion properties both into and out of the cell (Dudman, 1977 [10]). For instance, cells buried within a polymer matrix would be inaccessible to antibiotics. Anionic exopolysaccharide bind met-

als affecting the penetration of both useful and toxic ions through the cell surface. This type of interaction assumes practical importance in the corrosion of metallic surfaces. Moreover, EPS play an important role in bacterial growth on hydrocarbons. Bioemulsifiers are amphipathic proteins and/or polysaccharides that stabilise oil-in-water emulsions (Ronsenberg and Ron, 1997 [31]). Extracellular biosurfactants and bioemulsifiers increase oil/water interfaces enhancing substrate mass transfer and allowing more microorganisms to contact the hydrocarbon substrates. Emulsifiers increase the hydrocarbon/water interfaces stabilizing oil droplets in the water emulsion (Franzetti et al., In press [12]).

Source of exopolysaccharide-producing bacteria

Microorganisms producing exopolysaccharide are found in various ecological niches. Environments with high carbon/nitrogen ratio (e.g. effluents from the sugar, paper or food industries as well as wastewater plants and sewage sludge) are known to be naturally enriched in microorganisms producing polysaccharides. Moreover, microorganisms utilizing petroleum derivatives might be EPS-producers as some of them synthesis bioemulsifiers (Morin, 1998 [27]).

Isolation of exopolysaccharide-producing bacteria

Exopolysaccharide-producing microorganisms can be isolated using complex media or chemically defined synthetic ones. These organ-

isms produce colonies with mucoid or watery surface and thus can be detected macroscopically. However, Morin (1998) [27] stated that no direct correlation exists between morphological characteristics of the colonies on solid medium and the ability of a culture to produce polysaccharide in a liquid medium. Some polysaccharides form stable complexes with water-soluble dyes, such as aniline blue which could be used as a screening tool.

2.1.2 Production of exopolysaccharides

Microorganisms are used extensively to provide a vast range of products and services. They have proved to be particularly useful because of the ease of their mass cultivation, speed of growth, use of cheap substrates (which in many cases are wastes) and the diversity of potential products. Their ability to readily undergo genetic manipulation has also opened up almost limitless possibilities for new products and services from the fermentation industries. However, the development of any new fermentation product depends on many factors, including the market, the current level of scientific knowledge, and the regulatory environment being some of these factors out of the control of the developer (Waites et al, 2001 [40]).

Successful commercialisation of every biotechnological product depends largely on its bioprocess economics and various strategies have been developed to make biopolymer production process cost-

competitive. Currently, only a small number of biopolymers are produced commercially on large scale and among them, xanthan from *Xanthomonas campestris*, gellan from strains of *Sphingomonas paucimobilis*, dextran from *Leuconostoc mesenteroides* and emulsan produced by *Acinetobacter lwoffii* RAG-1 are the exopolysaccharides.

Development of a cost-efficient production process

Development of biotechnological process is composed of several stages: two sequential screenings, the downstream process optimisation and the evaluation of production cost (Figura 2.1).

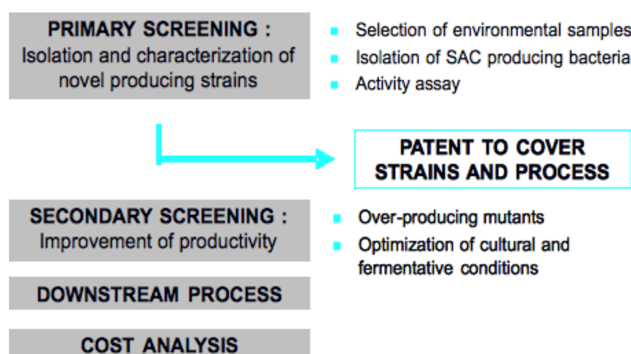

Figure 2.1: Development of biotechnological process

The primary screening is composed by the following steps: i) the selection of the environmental sample, ii) the isolation of microorganisms which produce the specific compounds of interest and iii)

the analysis of the compound activity. During the primary screening, the industrially-promising microorganisms are usually patented to protect them.

The aim of secondary screening is to improve the bioprocess productivity and several strategies are currently adopted (e.g. the use of overproducing mutants and recombinant strains).

The development of a complete biotechnological process also needs the optimisation of the procedure for the product recovery. Finally, the evaluation of the production cost allows determining the commercial potentiality of the bioproduct.

Presently, the use of bacterial exopolysaccharides, and microbial polymers in general, is limited by production costs. The production economy of every microbial metabolite is governed by three basic factors: i) initial raw material costs, (ii) availability of suitable and economic production and recovery procedures, and (iii) the product yield of the microbial producer. Three basic strategies were adopted worldwide to make biotechnological process cost-competitive: (i) the use of cheaper and waste substrates to lower the initial raw material costs involved in the process; (ii) the optimisation of the fermentative conditions and separation processes for maximum production and recovery, and (iii) the development and use of overproducing mutants or recombinant strains for enhanced bioproduct yields.

The cost of raw materials account for 10-30% of the overall pro-

duction costs which can be reduced by using low-cost substrates. The use of cheap and agro-based raw materials as substrates for biopolymer production has been extensively explored, including plant-derived oils, oil wastes, starchy substances, lactic whey and distillery wastes.

Several elements, media components and precursors have been reported to affect the biopolymer production process and the product final quantity and quality. Maximizing productivity or minimizing production costs demands on the use of process optimisation strategies that take into account multiple factors. The classical method of medium optimisation involves changing one variable at a time, while keeping the others at fixed levels; however, this method is laborious, time consuming and does not guarantee the determination of the optimal conditions for metabolite production. To tackle this problem and make the optimisation process easier, a statistical strategy, based on response surface methodology (RSM), has been used by various investigators. This method has been successfully used to determine the optimum medium composition, inoculum and cultural conditions for the enhanced production of many biopolymers (Mukherjee, 2006 [28]).

The use of cheap and waste substrate was successfully employed to promote the production of the extracellular polysaccharide by several bacterial strains. Freitas et al. (2009) [15] used glycerol

byproduct, generated by the biodiesel industry, in the production of an extracellular polysaccharide, with pseudoplastic fluid behaviour and emulsifying activity, by a strain of *Pseudomonas oleovorans*. Sugarcane molasses were employed in the production of EPS Gp CCT 7137, a water soluble acidic EPS with emulsifying activity for monoaromatic petroleum hydrocarbons, by *Gordonia polyisoprenivorans* CCT 7137 (Fusconi et al., 2010 [16]).

Bajaj et al. (2006) [3] used a statistical approach to optimize gellan gum production in *Sphingomonas paucimobilis* ATCC 31461 and compared soluble starch, glucose, lactose, maltose and sucrose as a carbon source. Recently, Response surface methodology was also used in order to optimise exopolysaccharide production in *Agrobacterium radiobacter* (Triveni et al., 2001 [38]), glucan production from *Leuconostoc dextranicum* (Majumder et al., 2009) and emulsan production of *Acinetobacter venetianus* RAG-1 (Su et al., 2009 [34]).

EPS production and recovery

Processes for production of exopolysaccharide are characterized by the extreme rheology of the fermentation, product concentration, the diversity of subtle structural and conformational changes (which can occur throughout the entire process) and the discernable effect of these changes on the products and application performance.

There is no single set of culture conditions that guarantees high exopolysaccharide yields, since organisms differ in their carbon and nitrogen source requirements, temperature and pH optima which are the critical factors for maximum exopolysaccharide production. The yield and quality of microbial exopolysaccharide are greatly affected by the nutritional and environmental conditions and an increase in polymer production is possible by manipulating the culture conditions.

The cost of recovery of exopolysaccharide, including concentration, isolation and purification, is a significant part of the total production cost. This is due to low concentration of exopolysaccharide in the fermentation broth, the presence of contaminating solids (e.g. cells), solutes in the stream, and the high viscosity of the fermentation liquid/broth. The constraint in downstream processing of exopolysaccharide lies in the separation of microbial cells from the culture broth and the degree of association of the exopolysaccharide to the microbial cells (i.e. as slime or a capsule) (Kumar at al., 2007 [21]).

Xanthan gum: EPS who gained market competition

Some microbial polysaccharides are commercially accepted, while others are at various stages of development (Table 2.1). Bacterial EPS which are currently commercial products are xanthan, dextran,

emulsan and gellans gum. By far the most commercially successful example of a microbial exopolysaccharide is xanthan gum.

Xanthan gum has discovered in the late 1950s by US scientists and is the first biopolymer produced industrially. The natural source of the polysaccharide came from a cabbage plant bacterium, known as *Xanthomonas campestris*. It was not until 1969 that the FDA issued the final approval for the use of xanthan gum in food products. The demand for xanthan gum produced by *X. campestris* has increased steadily every year and is estimated to grow continuously at an annual rate of 5-10%. Commercial production of xanthan gum uses glucose as the substrate. Generally, batch production is preferred over continuous fermentation due to superior performance of the batch conditions (Rosalam and England, 2006 [30]).

Diverse industrial applications are based on the ability of xanthan gum to dissolve in hot or cold water and yield a high viscosity, even at concentrations as low as 0.05%. Approximately 60% of the xanthan produced is used in non-food application (stabilizer for paint emulsions, carrier for fertilizers and herbicides, thickener for textile dyes, drilling lubrificant, as well as tertiary recovery in the oil industry and clay coatings for high-quality paper). Food applications involve roles as thickener, adhesive, binder in films and coatings, emulsifying agent and stabilizer. Currently, xanthan has almost a quarter of the American market for food thickeners. Approximately

Organism	Polysaccharide/biopolymer	Application
Pseudomonas aeruginosa and *Azotobacter vinelandii*	Alginate	As immobilization matrix for viable cells and enzymes, coating of roots of seedlings and plants to prevent desiccation, micro-encapsulation matrix for fertilizers, pesticides and nutrients, hypoallergic wound-healing tissue
Acinetobacter calcoaceticus	Emulsan	-same as above-
Sphingomonas paucimobilis	Gellan	For solidifying culture media, especially for studying marine microorganisms
Streptococcus equii and *Streptococcus zooepidemicus*	Hyaluronic acid	As replacer of eye fluid in ophthalmic surgery, in artificial tear-liquid, synovial
Xanthomonas	Xanthan (E 415)	In secondary and tertiary crude-oil recovery, in paints, pesticide and detergent formulations, cosmetics, pharmaceuticals, printing inks (to control viscosity, settling and gelation), in food as thickening and stabilizing agent, often used in combination with guar gum.
Acetobacter spp.	Cellulose	In human medicine as temporary artificial skin to heal burns or surgical wounds, in nutrition as natural non-digestable fibers (which can be impregnated with amino acids, vitamins and minerals), as hollow fibers or membranes for specific separation technology, as acoustic membranes in audio-visual equipment
Rhizobium meliloti and *Agrobacterium radiobacter*	Curdlan	As a gelling agent, immoblization matrix, Curdlan along with zidovudine (AZT), displays promising high antiretroviral activity (anti AIDS-drug)
Alcaligenes faecalis var. *myxogenes*	Succinoglycan	-same as above-
Leuconostoc mesenteriodes	Dextran	In veterinary medicine, in human medicine as blood plasma extender or blood flow improving agent and as cholesterol lowering agent, in separation technology, as molecular sieve and in aqueous two phase systems, as micro-carrier in tissue/cell culture (cross-linked dextran)

Table 2.1: Bacterial polymers, producing strains and their applications (Kumar at al., 2007 [21]).

20.000 tonnes of xanthan are produced each year (Waites et al, 2001 [40]).

2.1.3 *Gordonia* sp.BS29 as a producer of EPS with emulsifying properties

Owing to its potential as a biopolymer source of industrial interest, EPS-producing microorganisms have been isolated from a wide range of environments. Among them, *Gordonia* sp. BS29, was isolated as an hydrocarbon degrading bacteria from a diesel contamined soil and then characterised as an EPS-producing strain. The strain BS29 is able to produce and to release lipopolysaccharidic emulsifing agents when grew both in water-soluble and insoluble substrates (Franzetti et al., 2008 [14] and 2009 [13]). Microcosm experiments have demonstrated that the BS29 bioemulsifier is a promising washing agent for the remediation of hydrocarbon-contaminated soils (Franzetti et al., 2009 [13]).

For all these reasons, *Gordonia* sp. BS29 is considered as a suitable microorganism to be employed for bioemulsifier production.

2.2 Materials and Methods

2.2.1 Evaluation of renewable and waste substrates for BS29 bioemulsifier production

Growth conditions and culture preparation

Liquid cultures were prepared in Tryptic Soy Broth (TSB, per litre): casein (pancreatic digest) 17 g, soya peptone (papaic digest) 3 g, sodium chloride 5 g, dipotassium phosphate 2.5 g, dextrose 2.5 g. The cells were removed by centrifugation, washed twice and suspended in M1 minimal medium (per litre): K_2HPO_4 1.32 g, KH_2PO_4 1 g, NH_4Cl 0.81 g, $NaNO_4$ 0.84 g, $FeSO_4$ * $7H_2O$ 0.01 g, $MgSO_4$ * $7H_2O$ 0.419 g, $CaCl_2$ 0.03 g. Cultures were grown at 30°C in a rotary shaker at 250 rpm.

Bioemulsifier production

The cultures were prepared in 100 mL Erlenmeyer flasks containing 25 mL of M1 minimal medium and inoculated to an initial OD_{600} of 0.050. Each different carbon source (sodium citrate, n-hexadecane, corn oil, palm frying oil, peanut oil, rapeseed oil, soybean oil, sunflower oil, castor oil, linseed oil, waste frying oil and sugar-beet molasses) was filter sterilised and supplied at an initial concentration of 20.0 g/L. Cultures were grown at 30 °C in a rotary shaker at 250 rpm. After seven days, the emulsification activity was deter-

mined. The measures of bacterial growth on different carbon and energy sources were performed as follows: cell collection by filtration through 0.2 μm filters, detachment of cells from filters by vigorously shaking for 5 min, suspension on saline solution and determination of OD_{600}. All determinations were performed at least in duplicate.

Determination of emulsification activity as E24% index

Samples of whole culture broths (with cells) or culture filtrates (without cells) were used. The cells were removed from the cultures by filtration through 0.2-μm filters. A 3-mL sample was vortexed while the same amount of the oil phase was added drop by drop over 30 s in a glass graduated tube. After this, the tube was vortexed for additional 2 min. Kerosene was used as oil phase when not differently specified. All emulsions were prepared at room temperature. The mixture was allowed to settle for 24 h. The emulsification index (E24%) is given as the percentage of middle emulsion phase normalized to the total volume (Cooper & Goldenberg, 1987 [8]). All determinations were performed at least in duplicate (Figure 2.2).

The emulsion forming and stability capacity was evaluated for different concentrations of the BS29 bioemulsifier, prepared as described in paragraph *Recovery of BS29 bioemulsifier* (Section 2.2.3). A direct proportional between the E24% index and the bioemulsifier concentration was found up to 0.020 mg/mL; higher concentrations

were not investigated (Figure 2.3).

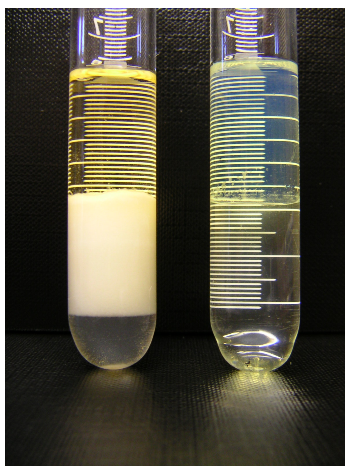

Figure 2.2: Determination of emulsifying activities by E24% emulsification assay.

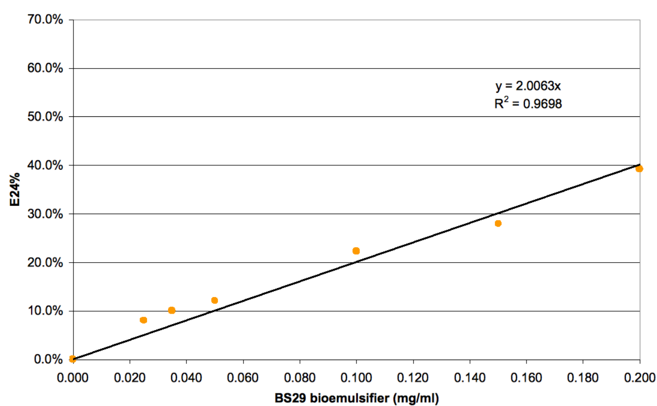

Figure 2.3: Relation between the E24% index and the concentration of the BS29 bioemulsifier (mg/mL) diluted in phosphate buffer 50 mM pH 7.2.

2.2.2 Optimization of emulsifiers production by *Gordonia* sp. BS29

Growth conditions and culture preparation

Liquid culture were prepared in TSB. The cells were removed by centrifugation, washed twice and suspended in physiological solution (NaCl 8.5 g/L). The cultures were prepared in 100 mL Erlenmeyer flasks containing 25 mL of each different cultural medium and inoculated to an initial OD_{600} of 0.050. Cultures were grown seven days at 30 °C in a rotary shaker at 250 rpm.

Determination of the responses

At the end of the seven-day incubation at 30 °C, the bacterial growth was measured by determining the optical density at 600 nm using an Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). The cultures were centrifuged at 6000 rcf for 15 min. Then, the supernatants were filtered through 0.2-μm filters. Aliquots of the culture filtrates were used for determination of E24% index and pH using WTW pH 720 (Weilheim, Germany) pH meter and WTW pH-Electrode Sentix41 (Weilheim, Germany). All determinations were performed at least in duplicate.

Determination of the emulsification activity by spectrophotometric assay (EA)

The sample was incubated overnight at 30 °C. The assay was carried out in a polypropylene tube. Fifteen μL of n-hexadecane were added

to phosphate buffer (50 mM; pH 7.2) and increasing amounts of the sample in a total volume of 3000 μL. After this, the tube was vortexed in VXR basic vibrax mixing orbital shaker (IKA) for 2 min at 2500 rpm at room temperature. The mixture was allowed to settle for 30 sec, then the turbidity was measured at 600 nm. One unit of emulsifying activity (UE) was defined as the amount of bioemulsifier that yielded an A_{600} of 0.1 in the assay. In the spectrophotometric emulsification assay, the emulsion turbidity was directly proportional to the concentration of the BS29 bioemulsifier up to 0.035 mg/mL (Figure 2.4).

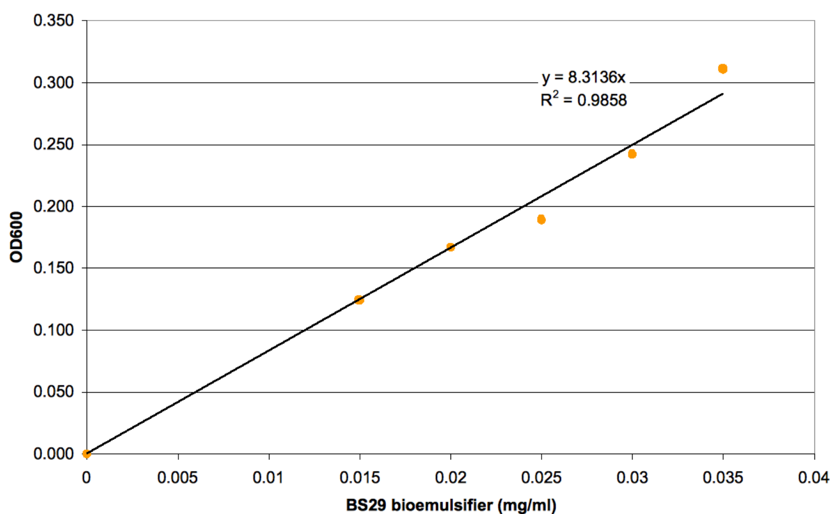

Figure 2.4: Emulsion turbidity (OD_{600}) versus concentration of the BS29 bioemulsifier (mg/mL) in the spectrophotometric emulsification assay.

Experimental Design by Response Surface Methodology

Preliminary screening experiments

The factors affecting the emulsifier production in *Gordonia* sp. BS29 were determined by a first screening design in batch cultivation at 100 mL shake flask scale. The cultures were prepared with 25 mL minimal medium. The type and concentration of the carbon source (sodium citrate or fructose) and the concentrations of the inorganic nutrients (buffer phosphate, NH_4Cl, $NaNO_3$, $FeSO_4$, $MgSO_4$) were chosen as factors for the analysis (Table 2.2 CaCl2* $2H_2O$ was employed in each experiment at the concentration of 29.4 mg/L.)

	-1	+1
Factor	x^{min}	x^{max}
Carbon source (g/L)	1	20
K_2PO_4/ KH_2PO_4 1:1 (mg/L)	400	4000
NH_4Cl (mg/L)	140	1400
$NaNO_3$ (mg/L)	150	1500
$FeSO_4$ * $7H_2O$ (mg/L)	1.5	15
$MgSO_4$ * $7H_2O$ (mg/L)	90	900

Table 2.2: Values of the higher (-1) and the lower (1) levels for each variable in the $2^{(6-2)}$ Fractional Factorial Design.

For the first screening experiment, a two level fractional factorial design (FFD) was chosen with a resolution of IV. For each carbon

source (sodium citrate or fructose), 19 experiments were thus performed (Figure 2.5).

The values of the dependent responses, E24% and EA (UE/mL), were analyzed using the trial version of "Modde" software (Umetrics, version 8.02).

Figure 2.5: Cultures of the $2^{(6-2)}$ Fractional Factorial Design with fructose as a carbon source.

Fermentor

Growth conditions and culture preparation were performed as described in paragraph *Growth conditions and culture preparation* (section 2.2.1). To scale up the system, a stirred 3-liter bioreactor (BIOFLO 110, New Brunswick Scientific, Edison, NJ, USA) was

used containing 1.7 L of M1 minimal medium supplemented with 20 g/L of a soluble carbon source (sodium citrate, fructose or mannitol). Batch fermentation was carried out at 30 °C under aerobic conditions obtained by air sparging and agitation speed of 240 rpm. OD_{600}, pH and E24% were monitored in the cell-free culture filtrates over time.

2.2.3 Properties of the partial purified BS29 bioemulsifier

Recovery of BS29 bioemulsifier

Growth conditions and culture preparation were performed as described in paragraph *Growth conditions and culture preparation* (section 2.2.1). The cultures were prepared in 1 L Erlenmeyer flasks containing 200 mL of M1 minimal medium. Cultures were inoculated to an initial OD_{600} of 0.050. Each different carbon and energy source (sodium citrate or fructose) was filter sterilised and supplied at an initial concentration of 20.0 g/L. Cultures were grown at 30 °C in a rotary shaker at 250 rpm. After 78 h, bacterial growth was measured spectrophotometrically at the wavelength of 600 nm.

Culture broth samples were centrifugated at 6.000 rcf for 30 min. After a freeze-drying concentration of the samples, the BS29 bioemulsifier in the supernatant was precipitated by the addition of ethanol 96 vol.% (3:1) and an overnight incubation at 4 °C. The

BS29 bioemulsifier was recovered by centrifugation (16.000 rcf, 30 min, 4°C), resuspended in deionised water and then dialysed (cellulose dialysis tube, molecular weight cut-off 12.400) against deionised water for 24 h. The retained solution was lyophilised and the dry weight determined. The BS29 bioemulsifier was resuspended in ultrapure distilled water (Gibco) at the concentration of 10 mg/mL and then stored at -20 °C.

Determination of emulsification activity

Surface tension was determined by the Du Noüy ring method using a 3S tensiometer (GBX, France). All determinations were performed at least in quadrupled.

2.2.4 Molecular analyses of *Gordonia* sp. BS29

Genomic DNA was extracted from bacterial cells by using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. For plasmid extraction, the procedure described by Kado and Liu (1981) [20] was used.

Genomic DNA (100 ng) was used as a template in a Ripetitive Extragenic Palindromic (REP-PCR) reaction using the single primer BOX-A1R (5' CTACGGCAAGGCGACGCTGACG 3') (Yamamura, 2004 [41]).

Amplified Fragment Length Polymorphism (AFLP) was carried out as previously described by Speijer et al. (1999) [33] and Vos et al. (1995) BIBLIO with slight modifications (Fanci et al., 2009 [11]). AFLP-PCR primers, EcoRI-0 (6- carboxyfluorescein - 5'-GACTGCGTACCAATTC) and MseI-0 (5' - GATGAGTCCTGAG-TAA), were chosen via a primer selection procedure aimed at obtaining a suitable number of well-resolved amplified fragments in a defined molecular weight range. Amplified fragments were separated by capillary electrophoresis on an ABI 310 bioanalyzer (Fanci et al., 2009 [11]). The sizes of AFLP fragments were determined by using a 50- to 400-bp internal standard (Rox 400HD) and Peack scanner software (version 1.0; Applied Biosystems). Only fragments ranging from 60 to 400 bp were analyzed.

2.3 Results and discussion

2.3.1 Development of a cost-efficient process for the bioemulsifier production by *Gordonia* sp. strain BS29

Gordonia sp. strain BS29 has been extensively characterised for its ability to produce extracellular lipopolysaccharides on water soluble and hydrocarburic compounds. The BS29 bioemulsifier is able to produce stable oil/water emulsions finding a potential application in soil remediation technologies (Franzetti et al., 2008 [14] and 2009 [13]). For all these reasons, *Gordonia* sp. BS29 may be a suitable microorganism to be employed for bioemulsifier production by fermentation process.

The strain BS29 has been attributed to the *Gordonia* genus by 16S gene sequence analysis (Franzetti et al., 2008 [14]). The phylogenetic position of the strain was updated based of the recent description of novel species within the genus. For the analysis, the 16S gene sequence of the BS29 strain (GenBank Accssion number EF064796) and those of all *Gordonia* type strains (retrieved from Ribosomal Database Project II database) were aligned by MULTALIN software (Corpet, 1988 [9]). A phylogenetic tree was inferred using the neighbour-joining method and the software MEGA version 4.0 (Kumar et al., 2008 [23]). Bootstrap analysis (1000 replicates) was used to test the topology of the neighbour-joining method data.

The strain BS29 was phylogenetically closely related to the species *G. lacunae* and *G. terrae* (Figura 2.6).

Bioemulsifier production on renewable and waste substrates

It has been estimated that raw materials account for 10 to 30% of the total production costs in most biotechnological processes. A strategy to reduce costs of microbial metabolites is the use of low-cost raw renewable materials (Mukherjee, 2006 [28]). In this work, a variety of cheap renewable substrates, including plant-polysaccharides, agro-industrial wastes (sugar-beet molasses), edible and non-edible plant-derived oils, and waste frying oil, was tested as substrates for the growth and bioemulsifier production by *Gordonia* sp. BS29 (Table 2.3).

The strain BS29 is able to grow efficiently on molasses, a wide spectrum of plant-oils, and on waste frying oil from food industry. However, it did not show a significant biomass increase on plant-polysaccharides (starch, carboxymethyl cellulose and pectin) after seven days.

The ability of whole culture broths (with cells) and culture filtrates (without cells) on renewable substrates to produce a stable kerosene-water emulsion was tested by determining the E24% index and compared with values obtained for cultures prepared in pure commercial compounds (potassium citrate and n-hexadecane). As

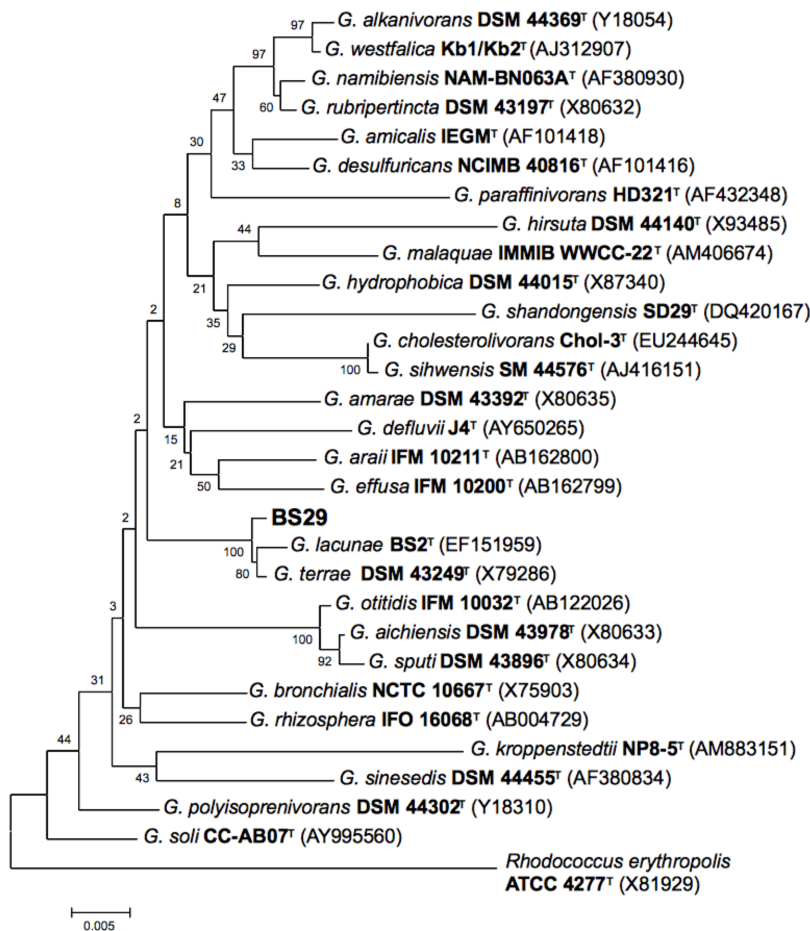

Figure 2.6: Unrooted phylogenetic tree based on 16S rRNA gene comparison. The positions of BS29 (EF064796) strain and the type strains of *Gordonia* species are shown. Bootstrap probability values that were less than 50% were omitted from the figure. The scale bar indicates substitutions per nucleotide position. The GenBank accession numbers are reported in parenthesis.

shown in Table 2.3, high emulsification activities were detected in whole culture broths and in cell-free filtrates when the strain was grown on molasses, a wide spectrum of plant-oils and waste frying oil.

Substrate (20 g/L)	**E24%**	
	C	**F**
Potassium citrate	73.3	35.0
Sugar-beet molasses	8.3	31.7
n-Hexadecane	39.6	44.0
Soybean oil	36.7	35.8
Palm frying oil	16.9	35.6
Waste frying oil	26.7	33.3
Corn oil	30.8	31.8
Linseed oil	25.0	31.4
Sunflower oil	47.1	30.1
Peanut oil	41.6	28.3
Rapeseed oil	21.7	28.3
Castor oil	0	10.7

Table 2.3: Emulsification activity (E24%) of whole culture broths (C) and cell-free culture filtrates (F) of *Gordonia* sp. BS29 grown on different renewable substrates. Values are means based on two separate experiments with two independent measurements each.

Overall, the *Gordonia* sp. strain BS29 displays important features for the future development of economically efficient industrial-scale biotechnological processes. It produces and releases extracel-

lular bioemulsifiers into the culture medium, which will simplify the recovery procedures. Furthermore, bacterial growth and production are supported by low cost renewable substrates. The use of cheap raw materials and wastes will contribute to the reduction of process costs. Consequently, our data encourage the development of the fermentation process.

In this work, the process development was started on water-soluble compounds due to the higher costs of plant management when immiscible compounds (hydrocarbons and oils) are used as fermentation substrate. Since the physiology of emulsifier biosynthesis in *Gordonia* is actually poorly elucidated, the study was preferentially carried out on commercial pure compounds. At research level, it is rare to use complex substrates as each one may contain a wide range of impurities and the composition of the substrate can be highly variable depending on what part of the world the substrate is grown in, soil type, climate, etc. It implies high in-process variability, even with careful planning of experimentation (e.g., using design of experiment software packages), and more replicate fermentations will be required to obtain clear results.

Flask-scale optimisation of medium composition

In order to evaluate the spectrum of carbon sources that the strain BS29 utilizes as substrate to produce bioemulsifiers, the strain was

grown on different hydrocarbons (n-C_{10-17}, branched alkanes and diesel) or water-soluble substrates (alcohol, organic acids, sugars). Among tested carbon sources, the strain did not exhibited a significant growth after seven days on arabinose, glucose, glycerol, maltose, pentaeritrol, xylitol, and xylose.

The ability of cell-free culture filtrates to produce stable kerosene-water emulsions was tested after a seven-day incubation (Table 2.4).

Carbon and energy source (20 g/L)	**E24%**
Sodium citrate	51.1
Fructose	50.4
Sodium acetate	45.0
Mannitol	40.8
Ethanol	39.0
Potassium citrate	35.0
Sorbitol	28.3
Sucrose	10.6
n-C10	64.9
Pristane	63.5
Diesel	63.4
n-C16	44.0

Table 2.4: Emulsification activity (E24%) of cell-free culture filtrates of *Gordonia* sp. BS29 grown on different carbon sources. Values are means based on two separate experiments with two independent measurements each.

On the basis of this analysis, the kinetics of *Gordonia* sp. BS29

were investigated on carbon sources (sodium citrate, fructose or mannitol) representative of different chemical classes (organic acids, sugars, and sugar alcohols) in batch cultivation using 0.5 L shake flasks.

Models allow understanding and describing characteristics of the system as a function of other system components and the cultural conditions. Models can be used in predictive mode and to design experiments, equipment and bioprocesses and to answer questions about the system (e.g. what will happen if we increase the temperature, decrease the concentration of carbon source). They can also involve the identification of the operating conditions for an economically breakeven point, for a given profit margin, etc. Bacterial growth was monitored over time by measuring the OD_{600} and the models describing the relationship between substrate concentration and specific growth rate was determined (Figure 2.7).

Comparative growth kinetics on sodium citrate, fructose and mannitol showed different behaviours (Figure 2.8). A saturation kinetics was the model describing bacterial growth on fructose and mannitol whereas the cultures followed substrate inhibition kinetics on citrate showing the maximum growth rate at 2.5 g/L. On stationary phase, the emulsification activity was determined by measuring the E24% index on the cell-free filtrates. Determinations were performed after 70 h for cultures on fructose and mannitol and 110 h

for those on citrate (Figure 2.9).

Based on obtained results, the range of carbon source concentrations was selected for the following optimisation step. Statistical experimental design methodology was used in order to investigate the cultural factors affecting the emulsifier synthesis by *Gordonia* sp. BS29 and to optimise the medium composition for the production.

A preliminary screening experiment was carried out to identify the factors and the interactions among factors having a significant influence on the emulsifier production (Table 2.5). The E24% index and the emulsification activity (EA) determined by spectrophotometric assay were the responses. Moreover, the optical density of the broth cultures and pH of the culture supernatants were determined.

In the preliminary experimental design, the concentrations employed showed no significantly results where sodium citrate was used as the carbon source. The citrate range needs to be reduced in order to investigate more accurately the substrate concentrations giving specific growth rates nearest to the maximum value.

On fructose, the most significant factors were the phosphate buffer concentration and the interaction between this factor and fructose concentration when the E24% index was used as response in the analysis (Table 2.6). However, the model did not shown a statistical significance.

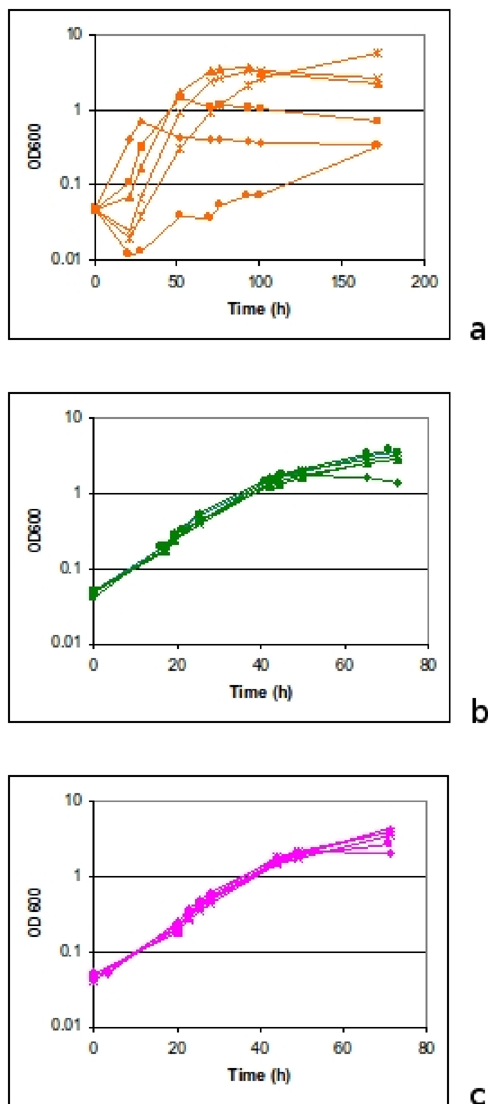

Figure 2.7: Kinetics of *Gordonia* sp. BS29 on citrate (a) fructose (b) or mannitol (c) in 0.5-L shake flasks.

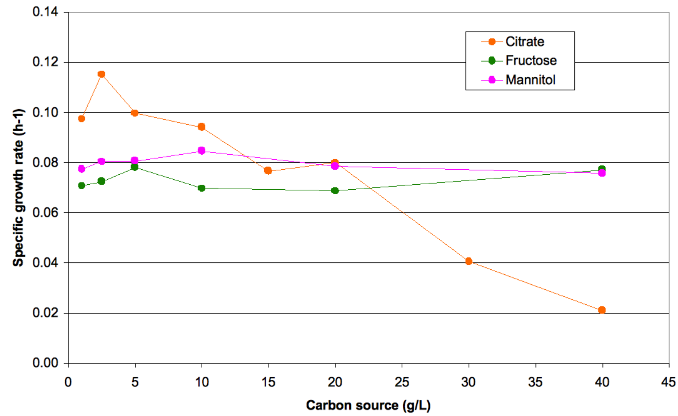

Figure 2.8: Effect of concentration of different carbon sources on the specific growth rate of *Gordonia* sp. BS29.

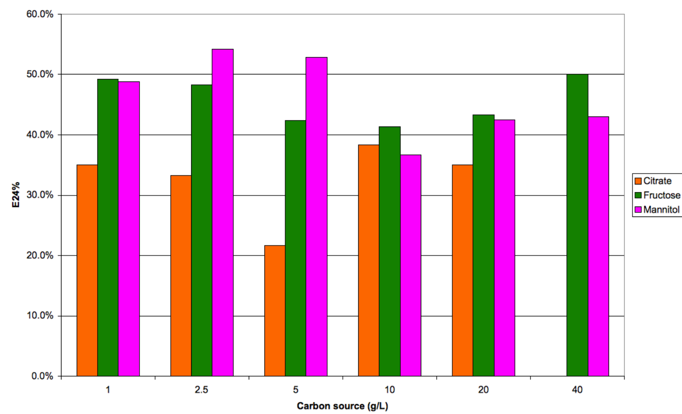

Figure 2.9: Emulsification activity (E24%) on cell-free filtrates of culture broths collected during the stationary phase of *Gordonia* sp. BS29 on citrate, fructose and mannitol.

From the two level fractional factorial design employing fructose as a carbon source, the concentrations of phosphate buffer, MgSO4, and fructose and the interaction among these factors, were the significant factors affecting the bioemulsifier production when the response was EA ((Figure 2.10) e (Table 2.6)). The output of the ANOVA test is shown in Table 2.7 and Table 2.8.

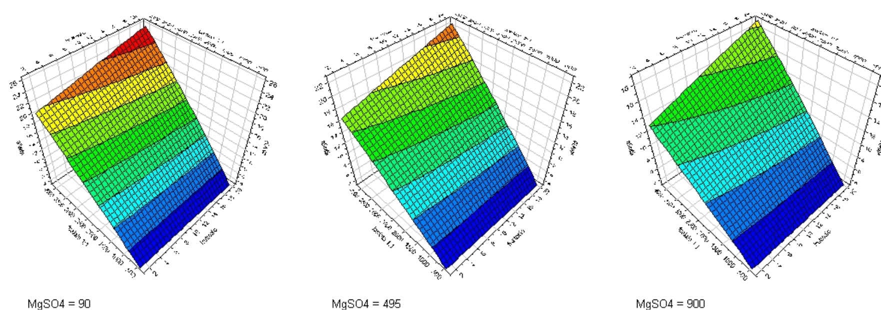

Figure 2.10: Response surfaces of the effect of the concentrations of phosphate buffer, $MgSO_4$, and fructose on the emulsification activity determined by spectrophotometric assay.

The next step in the optimisation procedure will be a second screening design where the phosphate buffer and fructose concentrations will be increased whereas the magnesium one will be decreased.

Experimental conditions						
Exp no.	**Carbon source**	**Phosphate**	**NH_4Cl**	**$NaNO_3$**	**$FeSO_4$**	**$MgSO_4$**
1	-1	-1	-1	-1	-1	-1
2	+1	-1	-1	-1	+1	-1
3	-1	+1	-1	-1	+1	+1
4	+1	+1	-1	-1	-1	+1
5	-1	-1	+1	-1	+1	+1
6	+1	-1	+1	-1	-1	+1
7	-1	+1	+1	-1	-1	-1
8	+1	+1	+1	-1	+1	-1
9	-1	-1	-1	-1	-1	-1
10	-1	-1	-1	+1	-1	+1
11	-1	+1	-1	+1	+1	-1
12	+1	+1	-1	+1	-1	-1
13	-1	-1	+1	+1	+1	-1
14	+1	-1	+1	+1	-1	-1
15	-1	+1	+1	+1	-1	+1
16	2.5	+1	+1	+1	+1	+1
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0

Table 2.5: Experiments performed in a first screening design.

Exp no.	OD_{600}	**pH**	**Response**	
			E24%	**EA(UE/mL)**
1	0.433	4.34	25.9	3.0
2	0.425	3.87	27.3	3.3
3	1.232	6.44	55.9	14.6
4	3.535	6.39	39.7	20.5
5	0.422	4.31	13.3	3.6
6	0.497	4.29	20.9	3.2
7	1.256	6.46	45.4	19.8
8	3.120	5.72	44.2	28.3
9	0.388	4.19	5.0	4.6
10	0.496	4.371	30.0	3.2
11	1.108	6.50	48.4	22.1
12	3.690	5.49	39.2	24.1
13	0.438	4.22	20.8	4.1
14	0.482	4.55	31.7	3.6
15	1.156	6.35	51.7	12.9
16	3.055	5.50	43.4	18.1
17	2.390	5.29	45.9	12.6
18	2.280	5.12	45.0	9.9
19	2.395	4.92	43.4	9.7

Table 2.6: Responses performed in a first screening design on fructose.

Activity	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int (±)**
Constant	-1,94432	0,00180	<0.0001	0,00390
Fru	0,00678	0,00197	0,00425	0,00425
Fos	0,04105	0,00197	<0.0001	0,00425
Mg	-0,00943	0,00180	<0.0001	0,00425
Fru*Fos	0,00792	0,00197	0,00142	0,00425
Fos*Mg	-0,00977	0,00197	<0.0001	0,00425

N=19	Q2=	0,949	Cond. no.=	1,09
DF=13	R2=	0,975	Y-miss=	0
	R2 Adj.=	0,966	RSD=	0,007865
			Conf. lev.=	0,95

Table 2.7: Results of the ANOVA test for the level fractional factorial design employing fructose as a carbon source.

Activity	DF	SS	MS (variance)	F	p	SD
Total	19	71,8594	3,78207			
Constant	1	71,8269	71,8269			
Total Corrected	18	0,03247	0,00180			0,04247
Regression	5	0,03167	0,00633	102,397	0,000	0,07958
Residual	13	0,00080	<0.0001			0,00786
Lack of Fit Model Error	11	<0.0001	<0.0001		0,607	0,00785
Pure Error	2	<0.0001	<0.0001			0,00792
Replicate Error						
	N= 19	Q2=	0,949	Cond. no.=	1,09	
	DF= 13	R2=	0,975	Y-miss=	0	
		R2 Adj.=	0,966	RSD=	0,007865	

Table 2.8: Results of the ANOVA test for the level fractional factorial design employing fructose as a carbon source.

Preliminary experiments in fermentor

Preliminary experiments were carried out in order to evaluate the bioemulsifier production in a 3-liter fermentor on the previously selected carbon sources (citrate, fructose and mannitol). OD_{600} and pH in the culture broths and E24% index in the cell-free filtrates were monitored over time. These parameters showed similar time-courses on mannitol (data not shown) and fructose. Over bacterial growth, a reduction in pH was observed in fructose whereas this parameter increased up to 8.79 on citrate (Figure 2.11). The biomass yields were 0.15, 0.24, and 0.35 g of dry weight per L on citrate, fructose and mannitol, respectively. On each tested carbon source, the E24% index increases during the exponential phase of growth reaching the higher values on the entering into the stationary phase. Moreover, the specific growth rate and the maximum emulsification activity obtained in fermentor was comparable to those at flask-scale. However, the formation of foam was evident at the end of the fermentations on carbon sources (Figure 2.12).

2.3.2 Emulsifying properties of BS29 bioemulsifier

The ability of the cell-free filtrates of *Gordonia* sp. BS29 grown on citrate (20 g/L) to emulsify different oil phases was tested. The bioemulsifier produced by the BS29 strain formed stable oil/water emulsion both with pure hydrocarbons (aliphatic, aromatic and

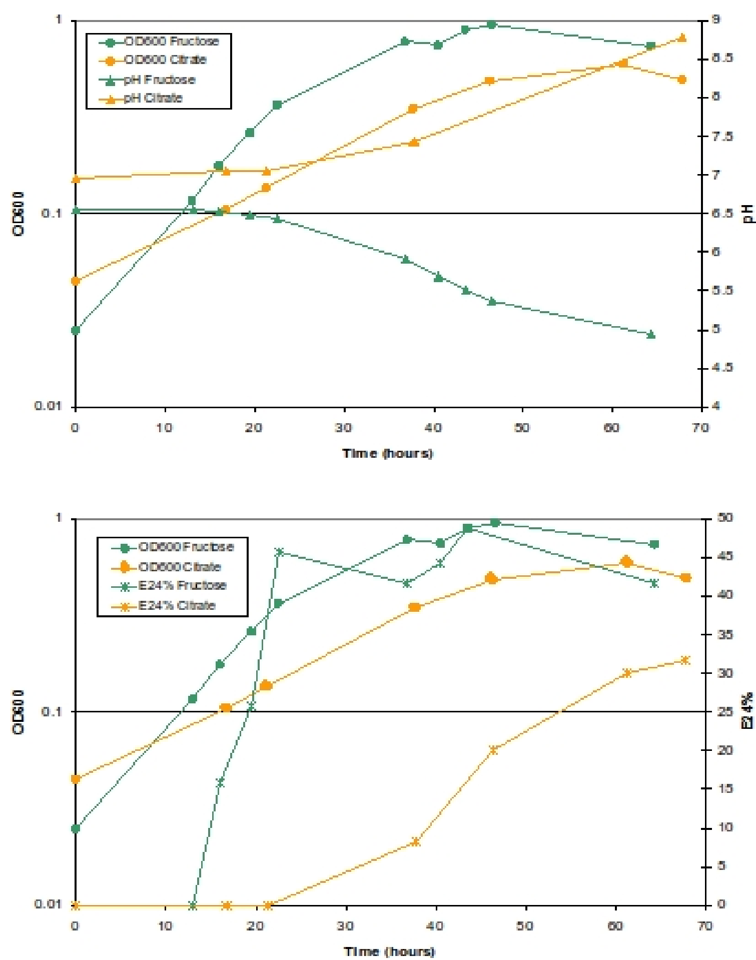

Figure 2.11: Kinetics of *Gordonia* sp BS29 on citrate (orange) or fructose (green) in 3-liter fermentor. • OD_{600}. ▲ pH ■ E24%.

Figure 2.12: Growth of *Gordonia* BS29 on citrate (a) or fructose (b) (20 g/L) in 3-liter fermentor.

cyclic compounds) and hydrocarbon mixtures (Table 2.9). Moreover, the surface tensions were measured in cultural supernatants and cell-free filtrates which did not displayed an efficient reduction of the surface tension compared to the M1 medium (Table 2.10).

In many industrial processes, emulsifiers are exposed to extremes of temperature, pressure, pH and/or ionic strength. Thus, the effect of pH, NaCl and heat treatments on the emulsifying activities of the BS29 bioemulsifier were evaluated by the spectrophotometric assay. The emulsifier was active from pH 3 to 9. Moreover, the EA increased 85% at pH 5 whereas a 5-fold increase was observed at pH 8 (Figure 2.13). The activity was almost constant up to 1 M NaCl showing a 50% decrease at 2 M NaCl (Figure 2.14).

The emulsifier demonstrated a noteworthy thermostability and

Oil phase	**E24%**
Cyclohexane	62.1
Kerosene	61.7
Toluene	59.3
n-hexane	46.7
Pristane	42.5
n-hexadecane	21.7
n-heptadecane	26.2
Diesel	18.3

Table 2.9: Emulsification activity (E24%) against different hydrophobic compounds of the cell-free culture filtrates of *Gordonia* sp. BS29 grown on citrate (20 g/L). Values are the means based on two separate experiments with two independent measurements each.

the EA increased 44% after heating at 60 °C (Figure 2.15). A similar characterisation has been previously performed by Toren et al. (2001) [37] on Alasan from *Acinetobacter radioresistens* KA53. As compared to Alasan, the BS29 bioemulsifier showed a lower thermostability and a superior stability at different pH.

The ability of the BS29 bioemulsifier to form stable kerosene/water emulsions was tested under different conditions of pH (3-9) and NaCl concentration (0-2 M). The presence of NaCl at concentrations higher then 1.0 M caused a 50% increase in the emulsification stability as determined by E24% index whereas the increase in pH caused a drastic decrease (Figure 2.16). The emulsion stability in-

Carbon source (20 g/L)	ST (mN/m)	
	C	**F**
Citrate	59.5 ± 5.2	62.6 ± 1.9
Fructose	59.5 ± 4.1	59.5 ± 4.1

Table 2.10: Surface tension (ST) of supernatants and cell-free filtrates of *Gordonia* sp. BS29 grown on different carbon sources (20 g/L). The ST of the sterile M1 medium was 73.3 ± 0.2 mN/m. Each value is the mean of four independent determinations.

dex (E24%) under different conditions is highly dependent on the oil phase that is to be emulsified. For example, the exopolysaccharide

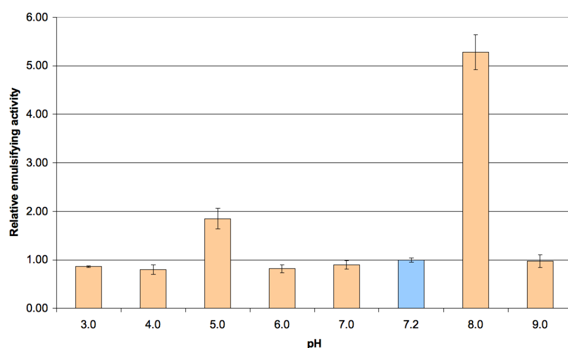

Figure 2.13: Effect of pH on the emulsifying activity of the BS29 bioemulsifier. The standard spectrophotometric assay was used except that the pH was varied by using different buffers (citrate buffer 50 mM for pH 3-5, phosphate buffer 50 mM for pH 6-8, and glycine buffer 50 mM for pH 9). Each value was the mean of at least three independent determinations. Values ± the standard errors relative to the activity determined in the standard assay (blue) are presented.

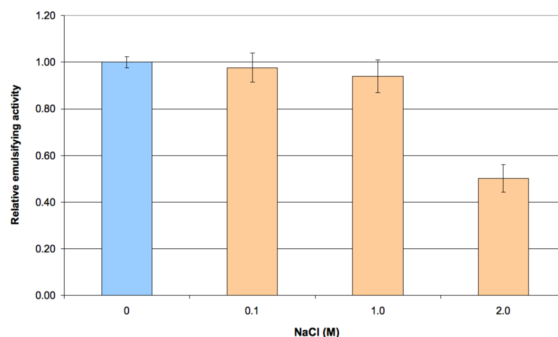

Figure 2.14: Effect of NaCl on the emulsifying activity of the BS29 bioemulsifier. Each value was the mean of at least three independent determinations. Values $\pm$ the standard errors relative to the activity determined in the standard assay (blue) are presented.

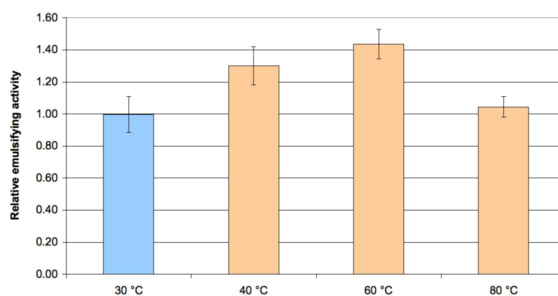

Figure 2.15: Effect of heat treatments on the emulsifying activity of the BS29 bioemulsifier. Each of the samples was heated at the indicated temperature for 10 min prior to the standard assay. Each value was the mean of at least three independent determinations. Values $\pm$ the standard errors relative to the activity determined in the standard assay (blue) are presented.

produced by *Enterobacter cloaceae* has shown to be able to emulsify hexane, being the emulsions stable for the pH range 2-10. By the contrary, the groundnut oil emulsion stability was decreased as the pH was increased from 2 to 10 (Iyer et al., 2006 [18]). *P. fluorescens* biosurfactant formed emulsions whose stability showed little changes by the presence of NaCl in concentrations up to 4.0 M (Abouseoud et al., 2008 [1]). The emulsions formed by the exopolysaccharide produced by *E. cloaceae* with hexane were stable in the presence of NaCl in the range of 0.1-1.0 M, but groundnut oil emulsions stability was improved by increasing the NaCl concentration (Iyer et al., 2006 [18]).

The capability of the BS29 bioemulsifier to form and stabilize emulsions was also tested after heat treatments for 10 min at 30 - 100 °C or for 15 min at 121 °C and 1 atm prior to the preparation of the emulsions (Figure 2.16). After cooling to room temperature, the emulsions were prepared with kerosene and left for 24 h to determine E24 index. The emulsion stability index decreased at increasing temperatures and a 40% of the activity was retained after autoclaving (Figure 2.16).

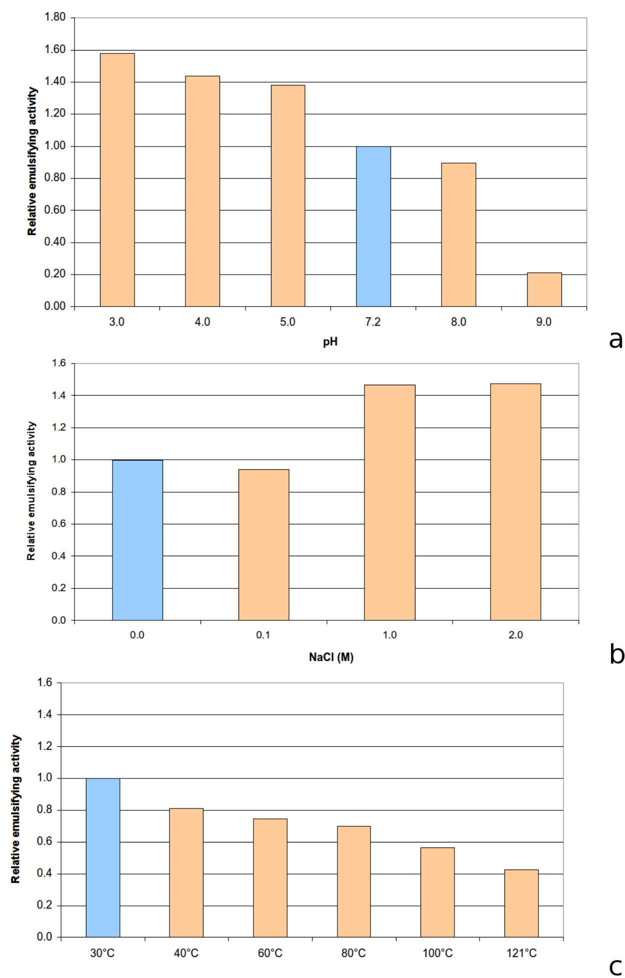

Figure 2.16: Influence of pH (a), NaCl (b) and heat treatment (c) on the emulsion stability index (E24%). Emulsions were formed between BS29 bioemulsifier aqueous solution (0.1 mg/mL).

2.3.3 Characterisation of the bioemulsifier-producing strain *Gordonia* sp. BS29 and its rough non-producing variant

One important goal during the development of an industrial process is the optimisation of a stable strain with constantly high productivity. Unfortunately, many producers change their phenotype during production condition (Zelder and Hauer, 2000 [43]). Although molecular mechanisms are not usually studied in detail, instability may result from genomic deletion and rearrangements both at plasmids and chromosomal level, which is referred to as structural instability, or complete loss of a plasmid, termed segregation instability. Production processes are situations of prolonged cultivation where selection pressure can hardly be maintained. After many generation times from cryo vials, agar plates, flasks, small fermenters to production fermenters one frequently detects mutant populations with improved growth rates and reduced production rates. The population will change if there is a selective advantage (McNeil and Harvey , 2008 [25]).

During the cultivation of the strain *Gordonia* sp. BS29 on tryptic soy agar (TSA), the colony showed a smooth morphology. However, rough morphological variants were frequently observed (Figure 2.17). This morphotype showed a constant phenotype also after repeated cultivation on solid rich medium. The smooth-to-rough con-

version of the colony morphology has been described in others members of the *Corynebacterineae* suborder and it is associated with the loss of the ability to synthesise amphiphilic compounds (Moorman et al., 1997 [26]; Iwabuchi et al., 2000 [17]). On the bases of these data, the ability of the rough variant to produce the extracellular emulsifier was investigated. A complete loss of the emulsification activity was observed when the variant was grown both on hydrocarbons and water-soluble carbon sources. Thus, the physiological and molecular characterisation of the *Gordonia* sp. BS29 and its morphological variant represents a fundamental activity for the development of the bioemulsifier production process and the elucidation its biosynthetic pathway.

The growth kinetics of the two morphotypes was compared on different carbon sources (citrate, fructose, and mannitol) and the relationships between substrate concentration and specific growth rate were determined (Figure 2.18). Models describing the kinetics of the rough non-producing variant were the same as previously described for the strain BS29 on all tested carbon sources. However, the specific growth rates were higher for rough morphological variant in investigated range of concentrations.

REP-PCR and AFLP analyses were performed in order to investigate the presence of genetic polymorphisms between the strain *Gordonia* BS29 and its rough non-producing variant.

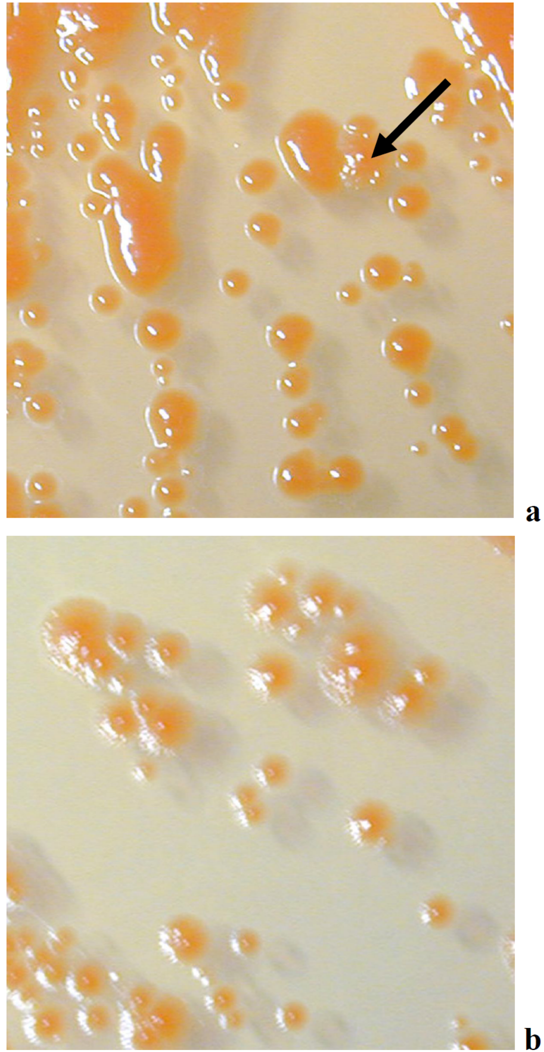

Figure 2.17: Colony morphology of *Gordonia* sp. BS29 (a) and its rough variant (b) on TSA.

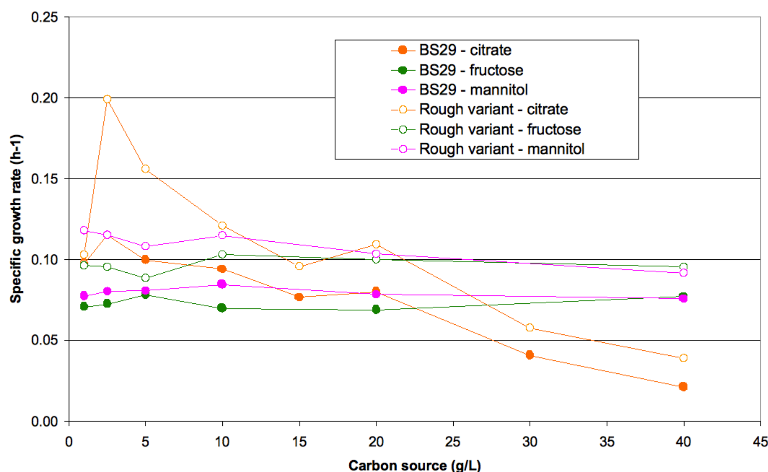

Figure 2.18: Effect of concentration of different carbon sources on the specific growth rate of *Gordonia* sp. BS29 and its rough non-producing variant.

The REP-PCR gives a genetic profile of a strain by the amplification of repetitive sequences disperse in the genome. REP have been characterized in several bacterial species and they are preferentially located within intergenic regions, in fact the REP frequency in these regions is 2.5 greater than in the rest of the genome. The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions for these highly conserved and dispersed elements. The comparison of REP profiles of the two morphotypes showed the presence of a 450-bp amplification product in the BS29 profile which was not detected in that of the rough variant. The band was extracted

from the gel and cloned in Escherichia coli by using the TOPO TA Cloning®(Invitrogen). Based on the sequence of the cloned fragment, a pair of specific primers was constructed. The subsequent amplification on genomic DNA of the two morphotypes showed the presence of the region also in the rough non-producing strain.

AFLP, developed by Vos et al. in 1995 [39], is a technique suitable to type both prokaryotic and eukaryotic species. It has high discriminatory power, it is reproducible and does not require knowledge of DNA sequences. Briefly, the stages to perform AFLP from a pure culture are: (i) DNA extraction, (ii) endonuclease restriction, (iii) fragment adapter ligation, (iv) selective amplification, (v) size separation of amplified fragments by capillary electrophoresis, and (vi) AFLP pattern analysis (Figure 2.19). AFLP requires a low amount of DNA, and often, it can be performed from low quality genomic DNA. The results are not affected by different DNA preparations or DNA extraction time.

To evaluate the reproducibility of the typing procedure, AFLP analysis was performed on three different samples of DNA obtained by independent extractions from the strain BS29 and its morphological variant. The comparison of AFLP profiles for each morphotype showed a high reproducibility of the AFLP procedure (data not shown). As shown in Figure 2.20, none polymorphic peak was identified by visual inspection of AFLP profiles of the two morphotypes.

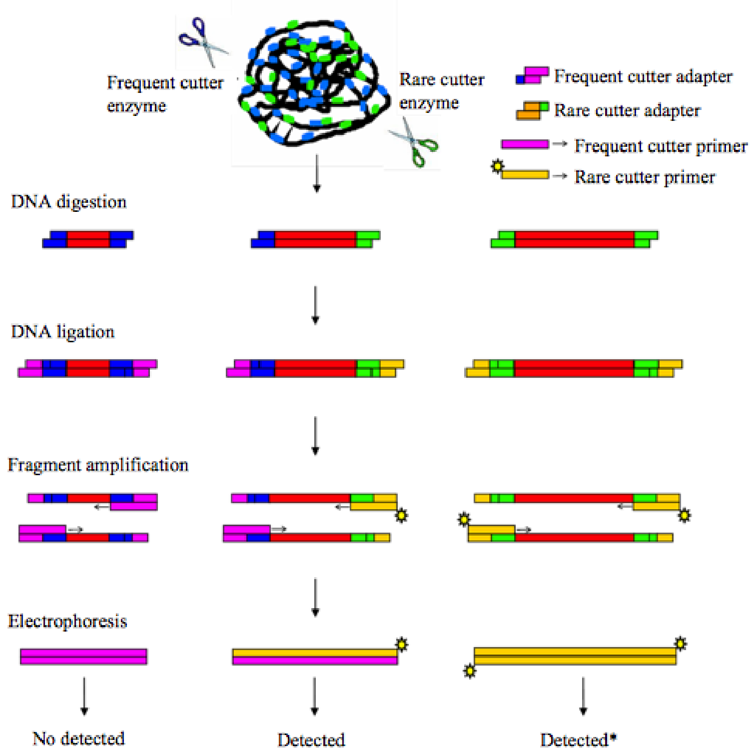

Figure 2.19: AFLP reaction. DNA is digested with two restriction enzyme, a frequent (blue) and a rare (green) cutter. Specific adapters are ligate to fragment ends. Fragments are amplified using specific primers, the rare cutter primer is fluorescent labelled. Fragments are separate on capillary electrophoresis. *Infrequent fragments, rarely detected.

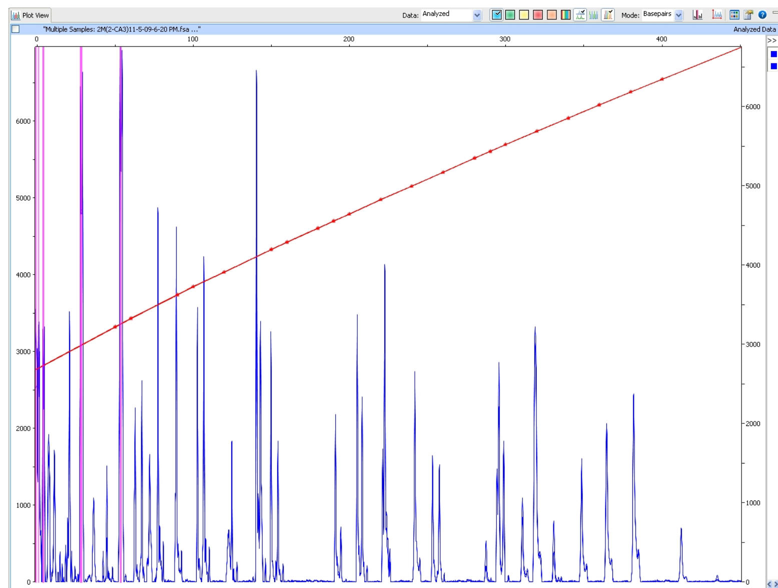

Figure 2.20: Overlay of the AFLP profiles of *Gordonia* sp. BS29 and its rough non-producing variant obtained by using the endonucleases *Eco* RI and *Mse* I.

The absence of polymorphisms in patterns generated by AFLP showed that mutations were not present in the *Eco* RI and *Mse* I restriction sites or in the sequences adjacent to these restriction sites and complementary to the selective primer extension, moreover insertions or deletions within the amplified fragments were not present. To better investigate the presence of genetic polymorphisms in the two morphotypes, future AFLP analyses will be performed using a different pair of restriction enzymes, such as *Apa* I and *Taq* I that recognizes DNA sequences of high G-C content (*Gordonia* G-C content ranges from 63 to 69 mol%). In this way it will be possible to modulate the number and the type of amplified fragments changing the selective primers and maximizing the polymorphism analysis (Janssen et al., 1996 [19]).

In order to evaluate if the non-producing phenotype of the rough variant is due to a segregational instability, the presence of (mega) plasmids in the strain BS29 was evaluated by the extraction procedure described by Kado and Liu (1981) [20]. The strain *G. westfalica* Kb1 harboring the megaplasmid pKB1 (101 Kbp) was used as control in the extraction. A megaplasmid was detected after electrophoresis of the DNA extracted from *Gordonia* sp. BS29 (Figure 2.21).

Noteworthy, the same is not demonstrable in the DNA obtained from the rough variant. However, the presence of the plasmid in the

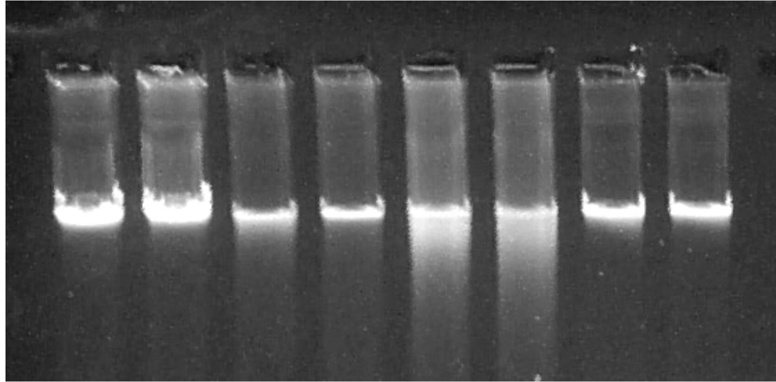

Figure 2.21: Electrophoresis of DNA extracted from *Gordonia* sp. BS29 (lines 1-2), the rough non-producing variant (lines 3-6), the *G. westfalica* Kb1 (lines 7-8).

rough variant can not be ruled out due to the lower quality of its DNA preparation compared to that extracted from the strain BS29.

The molecular and physiological characterisation of the bioemulsifier synthesis in *Gordonia* sp. BS29, described in this PhD Thesis, will be continued in the next two years since the Candidate has received a specific financing support to the Project "Genetica della biosintesi di emulsionanti batterici in *Gordonia*" by the *Autonomous Region of Sardinia* (Italy) in a Programme aimed to promote scientific research and technological innovation by Young Researchers ("Regional Law 7 of August 2007, n°7").

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