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REVIEW PAPER

Bacterial production of biosurfactants under microaerobic and anaerobic conditions

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Abstract Almost all knowledge about bacterial production of biosurfactants (BSFs) is limited to aerobic conditions. However, it is also known that bacteria can produce BSFs under oxygen-limiting conditions. These substances may be involved in important environmental processes (e.g. formation of gas hydrates and biofilms) or be applied in biotechnological processes (e.g. bioremediation and microbial enhancement of oil recovery, MEOR). Up to now, only few bacteria are described with the ability to produce BSFs under microaerobic and anaerobic conditions. Most of them belong to the *Bacillus* and *Pseudomonas* genera. However, BSF production under oxygen limitation has been detected in other bacterial groups (e.g. *Anaerophaga* and *Thermoanaerobacter*) involving different biosynthetic pathways. In this review, we summarize the current knowledge on growth requirements, cultivation conditions and properties of BSFs produced under oxygen-limiting conditions. In addition, we discuss the potential applications of microaerophilic and anaerobic BSF-producing bacteria in the perspective of bioremediation or MEOR strategies, energy and industry.

Keywords Anoxia · *Bacillus mojavensis* JF-2 · Microbe-enhanced oil recovery · Bioremediation · Gas hydrates

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1 Introduction

Surfactants are amphiphilic molecules composed of both hydrophobic and hydrophilic moieties. This allows them to concentrate at interfaces between phases and modify them in order to promote dispersion of one phase into the other. Surfactants are able to form aggregate structures, such as micelles, bilayers and vesicles (Van Hamme et al. 2006). Critic micellar concentration (CMC) is the minimum concentration in which surfactants in solution are able to spontaneously form micelles. Therefore this parameter is useful for comparing different surfactants, their activity and efficiency (Pacwa-Plociniczak et al. 2011). Surfactants with lower CMC values are considered to be more efficient since a smaller amount of surfactant is needed to decrease the surface tension (Desai and Banat 1997). In instances where surfactants are also able to produce emulsions, these compounds are also called emulsifiers (Trebbau de Acevedo and McInerney 1996) as they induce the formation of emulsions,

that is, the dispersion of one phase in another in the form of droplets (Nguyen et al. 2008).

Biosurfactants (BSFs) are molecules with identical properties to surfactants but produced by living organisms, such as bacteria. In microorganisms, BSFs are naturally involved in microbial competitive interaction (Van Alst et al. 2007), cell-to-cell communications (Hassett et al. 2002), plant and animal pathogenesis (Trejo-Hernández et al. 2014), increasing the bioavailability of surface-bound subtracts and heavy metals (via direct interfacial contact and pseudosolubilization; Elliot et al. 2010; Olaniran et al. 2013; Zhao et al. 2015e; Li et al. 2015), avoidance of toxic elements and compounds (Sandrin et al. 2000; Chrzanowski et al. 2011), motility (Okkotsu et al. 2013) and biofilm formation and maintenance (Ron and Rosenberg 2001; De Kievit 2009; Nickzad and Déziel 2014; Trejo-Hernández et al. 2014). Taking advantage of some of these characteristics, under biotechnological and industrial contexts, BSFs are used mainly as antimicrobial agents, detergents, emulsifiers, dispersants and foaming or wetting agents (Singh et al. 2007; Mandal et al. 2013). Their industrial applications are related to the pharmaceutical industry and production of cosmetics and personal care products, biological pest control and agriculture, food industry, bioremediation and microbial enhanced oil recovery (MEOR), mining and metallurgical industries (Singh et al. 2007; Mandal et al. 2013; Gudiña et al. 2013; Geys et al. 2014).

BSFs are particular advantageous when compared to synthetic surfactants due to lower toxicity and higher biodegradability (Bregnard et al. 1998; Mohan et al. 2006). These are the main reasons why they are preferred over synthetic surfactants in bioremediation and industrial applications. In general, BSFs also present better emulsifying properties and good stability at extreme pH, salinity and temperature values (Mukherjee 2007; Gudiña et al. 2012; Zhao et al. 2015c). However BSF production and competitive commercialization is still hampered by higher production costs, lower variety of produced BSFs and inefficient transfer of scientific knowledge to the industry (Geys et al. 2014).

Production of BSF in oxygen-limiting conditions occurs naturally in environmental conditions, such as during gas hydrates formation (Zhang et al. 2007), and can have application in biotechnological processes which may require the production of BSF in environments poor in oxygen, like MEOR (Perfumo et al. 2010). However, potential BSF-producing bacteria isolated from anaerobic or microaerobic environments, such as oil wells and sediments, are often only grown and tested for production of BSF under aerobic conditions due to time and material restrictions (Vasileva-Tonkova and Gesheva 2007; Fakhry et al. 2008; Mnif et al. 2011; Czajkowski et al. 2012; Mandal et al. 2013). In the context of this review, an environment will be considered as aerobic when molecular oxygen (O_2) corresponds to 21% or more of gaseous environments or there is over 30% oxygen saturation in aquatic environments. Microaerobiosis correspond to lower percentages of O_2 than in aerobic conditions, 1–30% of oxygen saturation in liquid media and <21% in air. Strict anaerobiosis is the complete lack of O_2. Microbes can be classified depending on their ability to grow under these conditions: obligate aerobes require O_2 for growth; facultative anaerobes can use several terminal electron acceptors including O_2 or operate fermentative pathways; microaerophiles can use O_2 but only in lower concentrations than those present in normal atmosphere/solutions, usually 1–15% in air or <30% saturation of oxygen in liquid media; aerotolerant anaerobes do not use O_2 as a terminal electron acceptor but can live in its presence; and strict or obligate anaerobes not only do not use O_2 but are also inhibited or even killed in its presence (Pelczar et al. 1993; Madigan et al. 2014).

Biosurfactant-producing bacteria have been extensively reported in scientific literature, especially under aerobic conditions, and several detailed reviews about the topic have already been published (Desai and Banat 1997; Satpute et al. 2010). However, relatively few studies addressed microaerobic and anaerobic conditions, even though the earliest mention of production of BSF under anaerobiosis was published in 1955 (La Rivière 1955). Since then few bacteria have been identified as being able to produce BSF under oxygen-limiting conditions, with most of these being described after the turn of the millennia. The present review intends to summarize and systematize the current knowledge on production of bacterial BSF under microaerobic and anaerobic conditions thus contributing to an in depth perspective on ecology of BSF-producing bacteria and their potential biotechnological and industrial applications.

2 Anaerobic and microaerobic biosurfactant-production: players and products

Few bacteria are known to produce BSFs under oxygen-limiting conditions. In this section, these bacteria are grouped by genera and relevant characteristics and the BSFs produced will be highlighted. *Bacillus mojavensis* JF-2, which is the most well studied anaerobic BSF producer, will be addressed separately as a biological model for anaerobic BSF production. A summary of bacterial isolates known to produce BSF in oxygen-limiting conditions can be found in Table 1 and respective culturing information in Table 2. Table 3 lists known properties of BSFs produced under microaerobic and anaerobic conditions.

2.1 *Bacillus mojavensis* JF-2

Bacillus mojavensis JF-2, previously known as *Bacillus licheniformis* JF-2 (Folmsbee et al. 2006) is able to produce the same type of surface active molecule, referred as BSF JF-2 or lichenysin B (Yakimov et al. 1995; Nerurkar 2010), under aerobic and anaerobic conditions. In both conditions, it lowers the surface tension of media by approximately 40 mN/m (Javaheri et al. 1985). In anaerobiosis, *B. mojavensis* JF-2 uses nitrate as primary terminal electron acceptor. Anaerobic cultures grow slower than aerobic ones but, in both cases, the BSF is produced and released during exponential phase of growth (Javaheri et al. 1985), being rapidly assimilated by *B. mojavensis* JF-2 cells at the beginning of the stationary phase (Lin et al. 1993). Uptake of BSF by cells during stationary phase is mediated by unknown cell surface components (Lin et al. 1993). The internalized BSF is thought not to be used as carbon or energy source and the uptake process is inhibited by magnesium ions (Lin et al. 1993). It has been hypothesized that the internalization of the BSF may be connected to a change in the development of the bacteria, possibly working as a chemical signal (Lin et al. 1993). Due to the narrow window of BSF production before it is taken up by stationary phase cells and because of the loss of ability to produce BSF that occurs after several generations in liquid cultures (Javaheri et al. 1985), large scale production of BSF by *B. mojavensis* JF-2 still presents major technical challenges. Although regarded as promising for MEOR strategies, *B. mojavensis* JF-2 is not particularly appealing for large scale BSF production, in comparison with other microorganisms with less complex BSF formation kinetics.

Lichenysin B is an anionic BSF with a molecular weight of 1035 Da and *B. mojavensis* JF-2 produces only one isoform (Lin et al. 1994b). Lichenysins are structurally very similar to surfactin (Fig. 1) and are sometimes classified as an isoform of the later (Stein 2005). Lichenysin B is a cyclic lipopeptide, composed of a heptapeptide structurally identical to surfactin (L-Glu–L-Leu–D-Leu–L-Val–L-Asp–D-Leu–L-Leu), with a β-hydroxy fatty acid amidated to the N-terminal amine of the peptide, while the C-terminal amino acid of the peptide is esterified to the β-hydroxy group of the fatty acid, forming a lactone ring (Fig. 1; Lin et al. 1994b; Konz et al. 1999; Youssef et al. 2005). The β-hydroxy group is mainly composed of either a normal, *iso*, or *anteiso* branched C_{14} or an *iso* or *anteiso* C_{15} (Yakimov et al. 1995). This results in a 'horse saddle' conformation, which is mostly responsible for the properties of the molecule, such as its antimicrobial activity (Peypoux et al. 1999; Sen 2010a). Both NaCl and Ca concentrations affect lichenysin B effectiveness. NaCl concentrations above 50 g/L are required to lower interfacial tension (<0.1 mN/m) and calcium concentrations above 25 g/L lead to an increase in surface tension (>2 mN/m), independently of temperature (Table 3; McInerney et al. 1990). Lichenysin B presents a CMC of 10 mg/L (Lin et al. 1994b), which makes it effective even in small concentrations and able to reduce interfacial tensions to very low values (<0.1 mN/m) (McInerney et al. 1990). Lichenysin B is stable when exposed to 25–120 °C for 20 min (Nerurkar 2010) and exhibits immunological and biochemical responses similar to surfactin, due to its almost identical hydrophilic moiety (Lin et al. 1994b). However, while surfactin is unstable in the presence of NaCl, lichenysin B is active in concentrations of up to 100 g/L (Nerurkar 2010).

2.2 Other *Bacillus* species

With the discovery and characterization of lichenysin B, close relatives of *B. mojavensis* JF-2 were soon screened for similar BSFs. Most studies were conducted in the presence of O_2 but the production of an analogue molecule, lichenysin A, was detected in *B. licheniformis* BAS50 cultivated in anaerobic conditions (Yakimov et al. 1995). *B. licheniformis* BAS50 is a facultative anaerobe isolated from an oil reservoir at

Table 1 BSF producing bacteria under microaerobic or anaerobic conditions and respective BSFs characteristics

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/L) (aerobic values)	Emulsion	Biofilm	Inoculum source	References
Anaerophaga thermohalophila Fru22 (DSM 12881)	Unidentified	25.9	NA	Yes	NA	Oil sludge and contaminated sedimentary residues from an oil separation tank; Hannover, Germany	Denger and Schink (1995) and Denger et al. (2002)
Bacillus amyloliquefaciens S499	Surfactin, iturin and fengycin	NA	252/108/10 (628/321/272)[a]	NA	Yes	Soil from Ituri, Congo	Nihorimbere et al. (2012)
Bacillus licheniformis BAS50	Lichenysin A	≈20 (26)	≈50 (155)	NA	NA	Oil from a reservoir at 1480 m depth; northen Germany	Yakimov et al. (1995, 1997)
Bacillus licheniformis BNP29	Unidentified	NA	≈35 (125)	NA	NA	Oil from a reservoir at a depth up to 1500 m; northen Germany	Yakimov et al. (1997)
Bacillus licheniformis BNP36	Unidentified	NA	≈8 (65)	NA	NA	Oil from a reservoir at a depth up to 1500 m; northen Germany	Yakimov et al. (1997)
Bacillus licheniformis Mep132	Unidentified	NA	≈45 (145)	NA	NA	Oil from a reservoir at a depth up to 1500 m; northen Germany	Yakimov et al. (1997)
Bacillus licheniformis VKM B-511	Unidentified	NA	NA	Yes	NA	Unknown	Gogotov and Miroshnikov (2009)
Bacillus mojavensis JF-2	Lichenysin B	≈41 (38)	34 (110)	Foam	NA	Well injection water; Oklahoma, USA	Javaheri et al. (1985), Lin et al. (1993, 1994a) and Folmsbee et al. (2006)
Bacillus strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049	Unidentified	≈17 (in reservoir)	≈350	NA	NA	Sahara desert, Tunisia	Youssef et al. (2007)
Bacillus subtilis ATCC 21332	Surfactin	≈40	439 (45)	Foam	NA	Unknown	Davis et al. (1999) and Zhang et al. (2007)
Bacillus subtilis BBG100	Mycosubtilin and surfactin	NA	≈70 (80)/≈20 (60)[a]	Foam	NA	Derivative of *B. subtilis* ATCC6633	Guez et al. (2008)
Bacillus subtilis C9 (KCTC 8701P)	C9-BS	(44.6)	4500 (7000)	Yes	NA	Unknown	Kim et al. (1997)

Table 1 continued

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/L) (aerobic values)	Emulsion	Biofilm	Inoculum source	References
Bacillus subtilis strain #191	Unidentified	24.7	NA	Yes	NA	Crude oil; Brazil	Gudiña et al. (2012)
Bacillus subtilis strain #309	Surfactin	35.0	NA	Yes	NA	Crude oil; Brazil	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis strain #311	Surfactin	35.9	NA	Yes	NA	Crude oil; Brazil	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis strain #552	Unidentified	21.8	NA	Yes	NA	Crude oil; Brazil	Gudiña et al. (2012)
Bacillus subtilis strain #573	Surfactin	34.9	NA	Yes	NA	Crude oil; Brazil	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis subsp. *spizizenii* ATCC 6633	Mycosubtilin and surfactin	NA	45 (2)/≈90 (115)[a]	NA	NA	Unknown	Guez et al. (2008)
Bretibacillus sp. BS2202	Unidentified	20.8 (22.7)	NA	NA	NA	Petroleum contaminated soil (30–40 cm deep); Noyabrskyi, Russia	Grishchenkov et al. (2000)
Clostridium pasteurianum sp.	Unidentified	NA	NA	NA	NA	NA	Cooper et al. (1980)
Desulfovibrio desulfuricans subsp. *desulfuricans* El Agheila Z (DSM 1926)	Unidentified	21	NA	NA	NA	Sulphurous mud; Ain-ez-Zania lake, Lybia	La Rivière (1955)
Geobacillus pallidus H9	Unidentified	35.48 (43.03)	NA	Yes	NA	Oil-containing brine; Daqing Oilfield, China	Wenjie et al. (2012)
Isolate Glc12	Unidentified	28.7	NA	Yes	NA	Oil sludge Petroleum contaminated sedimentary residues from a oil separation tank; Hannover, Germany	Denger et al. (2002)
Pseudomonas aeruginosa ATCC 10145	Rhamnolipid	≈28	≈100	Yes	NA	Unknown	Chayabutra and Ju (2000) and Chayabutra et al. (2001)
Pseudomonas aeruginosa E03–40	Rhamnolipid	NA	≈4500	Foam	NA	Soil samples; Iowa, USA	Pinzon et al. (2013)

Table 1 continued

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/L) (aerobic values)	Emulsion	Biofilm	Inoculum source	References
Pseudomonas aeruginosa PAO1 T	Rhamnolipid	NA	NA	NA	Yes	Unknown	Nozawa et al. (2007)
Pseudomonas sp. ANBIOSURF-1	Rhamnolipid	≈41	NA	Yes	NA	Municipal sewage sludge; location unknown.	Albino and Nambi (2010)
Pseudomonas sp. BS2201	Unidentified	14.5 (24.5)	NA	NA	NA	Petroleum contaminated soil (30–40 cm deep); Noyabrskyi, Russia	Grishchenkov et al. (2000)
Pseudomonas sp. BS2203	Unidentified	17.7 (21.3)	NA	NA	NA	Petroleum contaminated soil (30–40 cm deep); Noyabrskyi, Russia	Grishchenkov et al. (2000)
Pseudomonas aeruginosa WJ-1	Rhamnolipid	(≈45)	390 (50,200)	Yes	NA	Oil; Menggulin oil reservoir, China	Xia et al. (2012) and Zhao et al. (2015b)
Pseudomonas aeruginosa PrhlAB	Rhamnolipid	(37.2)	2420 (17,150)	NA	NA	Derivative of *P. stutzeri* SG, with plasmid pBBRPrhlAB	Zhao et al. (2015a)
Pseudomonas aeruginosa PoprAB	Rhamnolipid	(37.2)	3560 (20,980)	NA	NA	Derivative of *P. stutzeri* SG, with plasmid pBBRPoprAB	Zhao et al. (2015a)
Pseudomonas aeruginosa SG	Rhamnolipid	32.97 (37.2)	1080 (11,650)	Yes	NA	Water sample; Xinjiang oil reservoir, China	Zhao et al. (2015a, b, d, 2016a)
Pseudomonas stutzeri Rhl	Rhamnolipid	32.77	3120	Yes	NA	Derivative of *P. stutzeri* DQ1, with rhamnosyltransferase gene *rhlABRI* from *P. aeruginosa* SQ6	Zhao et al. (2014, 2015c)
Rhodococcus ruber Z25	Unidentified	NA	NA	Yes	NA	Formation brine; Daqing Oilfield, China	Zheng et al. (2012)
Thermoanaerobacter pseudethanolicus 39E (ATCC 33223)	Unidentified	NA	NA	Yes	NA	Hot springs microbial mat; Yellowstone National Park, USA	Yen et al. (1991)
Thermoanaerobacter sp. strains (mixed cultures)	Unidentified	NA	NA	NA	NA	Oil from carbonate reservoirs; Veracruz, Mexico	Castorena-Cortés et al. (2012)

NA not available

[a] Values corresponding to each BSF

Table 2 Conditions for BSF production by BSF-producing bacteria under oxygen-limiting conditions

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/L) (optimal)	pH	References
Anaerophaga thermohalophila Fru22 (DSM 12881)	Hexadecane	Glucose	Organic compounds	Anaerobic	37–55	20–120 (≈60[a])	6.7–6.8	Denger and Schink (1995) and Denger et al. (2002)
Bacillus amyloliquefaciens S499	NA	Glucose, fructose, maltose, ribose, citrate, succinate, malate and casamino acids	NO_3^-, organic compounds	Aerobic, anaerobic	28–30	0	6.8	Nihorimbere et al. (2012)
Bacillus licheniformis BAS50	$NaNO_3$	Glucose, sucrose	NO_3^-	Aerobic, anaerobic	25–50 (40–45)	0–130 (50)	5.4–8.5	Yakimov et al. (1995, 1997)
Bacillus licheniformis BNP29	$NaNO_3$	Sucrose	NO_3^-	Aerobic, anaerobic	25–55 (45–50)	0–130 (50–70)	6.0–6.7	Yakimov et al. (1997)
Bacillus licheniformis BNP36	$NaNO_3$	Sucrose	NO_3^-	Aerobic, anaerobic	25–55 (45–50)	0–130 (50–70)	6.0–6.7	Yakimov et al. (1997)
Bacillus licheniformis Mep132	$NaNO_3$	Sucrose	NO_3^-	Aerobic, anaerobic	25–55 (45–50)	0–130 (50–70)	6.0–6.7	Yakimov et al. (1997)
Bacillus licheniformis VKM B-511	Glutamic and aspartic acids	Glucose, sucrose, fructose, kerosene	NO_3^-	Aerobic, anaerobic	20–40 (30)	0–90 (90)	7.0–7.2	Gogotov and Miroshnikov (2009)
Bacillus mojavensis JF-2	DNA	Glucose	NO_3^-	Aerobic, anaerobic	40–50	20–100	4.6–9.0	Javaheri et al. (1985), Lin et al. (1993, 1994a) and Folmsbee et al. (2004, 2006)
Bacillus strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049	Addition of a nutrient mix in oil wells	Glucose, sucrose	NO_3^-	Aerobic, anaerobic	28–37	50	7.4–7.6	Youssef et al. (2007)
Bacillus subtilis ATCC 21332	NA	Glucose	NO_3^-, NO_2^-	Aerobic, anaerobic	20–32	0–50	7.0	Davis et al. (1999) and Zhang et al. (2007)
Bacillus subtilis BBG100	NA	Glucose	NA	Aerobic, microaerobic	30	0	6.0–7.0	Guez et al. (2008)
Bacillus subtilis C9 (KCTC 8701P)	NH_4HC0_3 and $MnSO_4$	Glucose, soybean oil, n-hexadecane	Organic compounds	Aerobic, microaerobic	30	0	6.8–8.0	Kim et al. (1997)

Table 2 continued

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/L) (optimal)	pH	References
Bacillus subtilis strain #191	NA	Tryptone, sucrose, n-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40–45 (up to 55 in solid medium)	10–100	7.0	Gudiña et al. (2012)
Bacillus subtilis strain #309	NA	Tryptone, sucrose, n-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40–50 (up to 55 in solid medium)	10–100	7.0	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis strain #311	NA	Tryptone, sucrose, n-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40–50 (up to 55 in solid medium)	10–100	7.0	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis strain #552	NA	Tryptone, sucrose, n-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40–45 (up to 55 in solid medium)	10–100	7.0	Gudiña et al. (2012)
Bacillus subtilis strain #573	NA	Tryptone, sucrose, n-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40–45 (up to 55 in solid medium)	10–100	7.0	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis subsp. *spizizenii* ATCC 6633	NA	Glucose	NA	Aerobic, microaerobic	30	0	6.0–7.0	Guez et al. (2008)
Bretibacillus sp. BS2202	P-limiting media with proteose peptone	Arabian light crude oil, glucose	NO_3^-	Aerobic, anaerobic	28	0–1	7–7.2	Grishchenkov et al. (2000)
Clostridium pasteurianum sp.	NA	Sucrose	NA	Anaerobic	NA	NA	NA	Cooper et al. (1980)
Desulfovibrio desulfuricans subsp. *desulfuricans* El Agheila Z (DSM 1926)	NA	Na-lactate	SO_4^{2-}	Anaerobic	30	10	7.0	La Rivière (1955)
Geobacillus pallidus H9	NA	Liquid paraffin, crude oil	NO_3^-	Aerobic, anaerobic	50–80 (65–70)	0–150 (<30[a])	6.8–7.2	Wenjie et al. (2012)
Isolate Glc12	Hexadecane	Glucose	Organic compounds	Anaerobic	37–55	20–120 (20–60[a])	6.7–6.8	Denger et al. (2002)

Table 2 continued

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/L) (optimal)	pH	References
Pseudomonas aeruginosa ATCC 10145	P-limiting media	n-Hexadecane, palmitic acid, stearic acid	NO_3^-, NO_2^-	Aerobic, anaerobic	22–35	0.5–5	6.2–7	Chayabutra and Ju (2000) and Chayabutra et al. (2001)
Pseudomonas aeruginosa E03–40	Betaine (osmopre -tectant)	Glycerol, tryptone	NO_3^-, NO_2^-	Aerobic, microaerobic	34	0.5	7.1	Pinzon et al. (2013)
Pseudomonas aeruginosa PAO1 T	8% CO_2 (in anaerobic conditions)	Tryptone, glycerol	NO_3^- NO_2^-	Aerobic, anaerobic	30–37	0–10	7	Nozawa et al. (2007)
Pseudomonas sp. ANBIOSURF-1	NA	Coconut oil	NO_3^-	Anaerobic	30	0–5	7.0	Albino and Nambi (2010)
Pseudomonas sp. BS2201	P-limiting media with proteose peptone	Arabian light crude oil, glucose	NO_3^-	Aerobic, anaerobic	28	0–1	7–7.2	Grishchenkov et al. (2000)
Pseudomonas sp. BS2203	P-limiting media with proteose peptone	Arabian light crude oil, glucose	NO_3^-	Aerobic, anaerobic	28	0–1	7–7.2	Grishchenkov et al. (2000)
Pseudomonas aeruginosa WJ-1	$NaNO_3$	Glucose, paraffin, glycerol	NO_3^-	Aerobic, anaerobic	(37)	1	6.0–8.0	Xia et al. (2012) and Zhao et al. (2015b)
Pseudomonas aeruginosa PrhlAB	$NaNO_3$	Glycerol	NO_3^-	Aerobic, anaerobic	37	1	6.8	Zhao et al. (2015a)
Pseudomonas aeruginosa PoprAB	$NaNO_3$	Glycerol	NO_3^-	Aerobic, anaerobic	37	1	6.8	Zhao et al. (2015a)
Pseudomonas aeruginosa SG	$NaNO_3$, PO_4^{3-}, SO_4^{2-}, Mg^{2+}	Glycerol, soybean oil, sunflower oil	NO_3^-	Aerobic, anaerobic	25–42	0–30	6.0–9.0	Zhao et al. (2015a, b, , d,2016a, b)
Pseudomonas stutzeri Rhl	KH_2PO_4	Glycerol, glucose, sucrose, molasses	NO_3^-	Aerobic, anaerobic	42	(1)	6.5–6.8	Zhao et al. (2014, 2015c)
Rhodococcus ruber Z25	$NaNO_3$	Liquid paraffin, crude oil	NO_3^-	Aerobic, anaerobic	37	0	7.5	Zheng et al. (2012)

Table 2 continued

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/L) (optimal)	pH	References
Thermoanaerobacter pseudethanolicus 39E (ATCC 33223)	NA	Glucose, tryptone	SO_4^{2-}	Anaerobic	65–70	12	NA	Yen et al. (1991)
Thermoanaerobacter sp. strains (mixed cultures)	NA	Molasses	Organic compounds	Anaerobic	50–80	5–35	7	Castorena-Cortés et al. (2012)

NA not available

[a] Values relative to biomass production (effect on BSF production was not tested/reported)

1500 m of depth, capable of reducing nitrate and producing spores only under aerobic conditions. Similarly to *B. mojavensis* JF-2, aerobic growth of *B. licheniformis* BAS50 resulted in a shorter lag phase, higher biomass production and lower surface tension than anaerobic cultivation, with minimum values of surface tension of 28.3 and 35 mN/m for aerobic and anaerobic conditions, respectively. In both conditions, the stationary phase was achieved within the same time. BSF, identified as lichenysin A, was produced throughout the exponential phase in aerobic and anaerobic conditions. Lichenysin A is structurally very similar to lichenysin B (Fig. 1), differing in the amino acid sequence, Glx-Leu-Leu-Val-Asx-Leu-Ile, and the β-hydroxy fatty acid which ranges from C_{12} to C_{17} normal, *iso* and *anteiso* forms, with the most common being *iso* C_{15} (38.6%). Additionally, lichenysin A has antimicrobial properties, albeit being less effective than surfactin (Yakimov et al. 1995).

Other nitrate-reducing strains (nitrate-reducing bacteria, NRB) of *B. licheniformis*, namely strains BNP29, BNP36 and Mep132 isolated from the same oil reservoir as *B. licheniformis* BAS50, were also able to produce BSFs under anaerobic conditions (Yakimov et al. 1997). Although less productive than *B. licheniformis* BAS50 (Table 1), they were also able to produce an extracellular polymer. When cultivated in anaerobic conditions, strains BNP29, BNP36 and Mep132 displayed a longer lag phase and shorter exponential and stationary phases. BSF production was identical to the *B. licheniformis* BAS50, and all strains produced larger amounts of BSF and smaller amounts of polymer in aerobic conditions, in comparison to anaerobiosis (Yakimov et al. 1997). The linkage between the biochemical pathways of BSF and polymer is still not understood but is possible that the lower yield of BSF in anaerobic conditions is linked to a diversion of carbon to biosynthetic pathways of polymer production.

In a screening of isolates with MEOR potential, *B. mojavensis* and *B. licheniformis* strains able to produce BSFs under anaerobic conditions in the presence of 50 g/L NaCl were detected (Youssef et al. 2005). In later studies testing the feasibility of a MEOR strategy, *Bacillus* RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049, both NRB, were able to produce BSF under anaerobic conditions, and even in the oil well environment (Youssef et al. 2007, 2013). Although oxygenation conditions inside the wells were not

Table 3 Characteristics of BSFs produced in microaerobic or anaerobic conditions

Bacteria	Biosurfactant	CMC (mg/L) (aerobic values)	BSF activity tolerance to NaCl (g/L) (optimal)	BSF activity tolerance to temperature (°C)	BSF activity tolerance to pH (optimal)	References
Anaerophaga thermohalophila Fru22 (DSM 12881)	Unidentified	NA	NA	<70 (20 min)	(2)	Denger and Schink (1995) and Denger et al. (2002)
Bacillus licheniformis BAS50	Lichenysin A	12	<300 (<100)	NA	NA	Yakimov et al. (1995)
Bacillus mojavensis JF-2	Lichenysin B	10	50–100	30–50	NA	Javaheri et al. (1985), McInerney et al. (1990), Lin et al. (1994b) and Folmsbee et al. (2006)
Bacillus subtilis C9 (KCTC 8701P)	C9-BS	40 mM	<58	20–100 (1 h)	4.0–10.3 (5.0–9.5)	Kim et al. (1997)
Bacillus subtilis strain #191	Unidentified	130	10–200 (50)	121 (20 min)	4–13 (6)	Gudiña et al. (2012)
Bacillus subtilis strain #309	Surfactin	20	10–200 (50)	121 (20 min)	4–13 (6)	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis strain #311	Surfactin	20	10–200 (50)	121 (20 min)	4–13 (6)	Gudiña et al. (2012) and Pereira et al. (2013)
Bacillus subtilis strain #552	Unidentified	100	10–200 (50)	121 (20 min)	4–13 (6)	Gudiña et al. (2012)
Bacillus subtilis strain #573	Surfactin	30	10–200 (50)	121 (20 min)	4–13 (6)	Gudiña et al. (2012) and Pereira et al. (2013)
Geobacillus pallidus H9	Unidentified	22 (16)	NA	NA	NA	Wenjie et al. (2012)
Pseudomonas sp. ANBIOSURF-1	Rhamnolipid	52	NA	NA	NA	Albino and Nambi (2010)
Pseudomonas aeruginosa SG	Rhamnolipid	80	<150	4–121	2.0–10.0	Zhao et al. (2015b)
Pseudomonas stutzeri Rhl	Rhamnolipid	90	<180	25–121	2.0–8.0	Zhao et al. (2015c)
Rhodococcus ruber Z25	Unidentified	57	NA	NA	NA	Zheng et al. (2012)

NA not available

described, they are expected to correspond to a microaerobic or anaerobic environment (Perfumo et al. 2010). Oil production was stopped for 108 h after bacteria inoculation and addition of nutrients. The average concentration of BSF in the inoculated oil wells when pumping was restarted was 90 mg/L, and a maximum of 350 mg/L was reached at approximately 10 h of pumping. In the inoculated oil wells, other non-spore forming bacteria, supposedly originated from the non-sterilized tanks where inoculum and nutrients were mixed, were able to produce BSF when a nutritional supplement, composed of glucose and mineral salts, was added to the wells. However, after 108 h of incubation in the oil wells, only the two inoculated *Bacillus* strains were recovered and confirmed as still able to produce BSF. Most metabolites detected in inoculated wells after 140 h of incubation were related to the anaerobic use of glucose. In laboratory experiments under anaerobic conditions a consortium of both *Bacillus* strains presented a growth

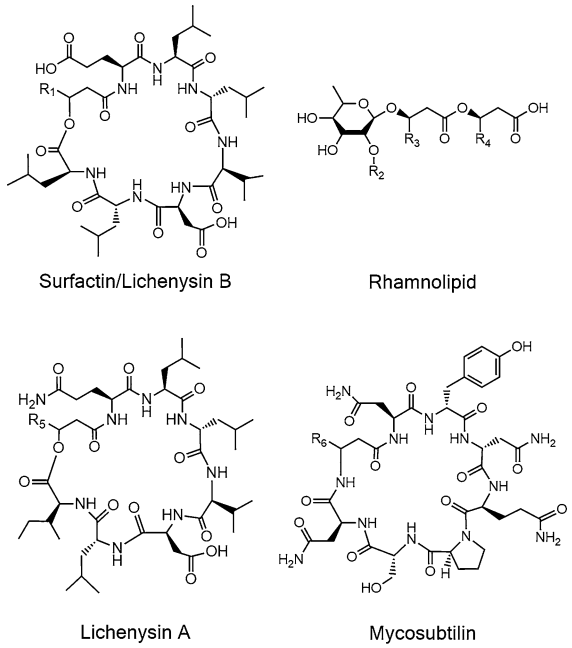

Fig. 1 Generic chemical structures of main BSFs produced in oxygen-limited conditions by bacteria. R_1 C_{12}–C_{16} β-hydroxy fatty acid in surfactin and C_{12}–C_{17} in lichenysin B (Yakimov et al. 1995). R_2 a hydrogen atom or α-L-rhamnopyranosyl. R_3 and R_4 C_8–C_{16} β-hydroxy fatty acid (Nitschke et al. 2005; Abdel-Mawgoud et al. 2010). R_5 C_{12}–C_{17} β-hydroxy fatty acid (Yakimov et al. 1995). R_6 C_{15-17} β-hydroxy fatty acid (Fickers et al. 2008)

rate of 0.18 h^{-1} and BSF yield of 0.02 mol BSF/mol glucose, while in inoculated oil wells, the respective values are 0.06 h^{-1} and 0.02 mol BSF/mol glucose (Youssef et al. 2007). Thus, while slower growth is observed in in situ conditions, BSF yields remain the same as in laboratory conditions.

In a search for strains able to produce BSF in oil reservoir conditions, five *B. subtilis* isolates (#191, #309, #311, #552 and #573) retrieved from crude oil, were tested (Gudiña et al. 2012). All strains were thermo- and halotolerant and able to grow and produce BSFs under both aerobic and anaerobic conditions, by using nitrate as terminal electron acceptor and fermentative pathways. BSFs showed good emulsifying activity, with the ability to reduce the medium surface tension below 40 mN/m (Table 1) and presented CMC values between 130 and 20 mg/L (Table 3). BSF produced by strains #309, #311 and #573 in aerobiosis, were identified as isoforms of surfactin with hydroxy fatty acid chains between C_{13} and C_{14} (Pereira et al. 2013).

A comprehensive experimental approach aiming the optimization of BSF production by the NRB *B. licheniformis* VKM B-511 (DSM 13) reported enhanced BSF production yields under anaerobiosis, resulting in an optimized yield of 4.58 g/L. Higher BSF yields and BSFs with higher emulsification indexes and decreased surface activity were obtained in anaerobic cultures when compared to aerobic conditions (Gogotov and Miroshnikov 2009).

Surfactin, one of the more studied and better known BSFs, is a secondary metabolite of the NRB *Bacillus subtilis* ATCC 21332. It is produced since early exponential phase but reaches maximum yields during stationary phase. Studies on the optimization of surfactin production showed that nitrate-limited anaerobic conditions can increase the BSF yield on biomass to 0.075, compared to only 0.021 in aerobic N-limiting conditions (Davis et al. 1999). Surfactin production by *B. subtilis* ATCC 21332 under anaerobic conditions is relatively fast, when compared with other bacteria. At 20 °C, BSF can be produced within approximately 24 h, which correspond to the beginning of its exponential phase (Zhang et al. 2007). Surfactin is structurally very similar to lichenysin B (Fig. 1), differing in its β-hydroxy fatty acids which can present normal, *iso* or *anteiso* C_{12}–C_{16} fatty acid chains, being *n* C_{14} predominant (40%) (Yakimov et al. 1995). The difference in nomenclature is due to the producing organisms, with lichenysins being produced by *B. licheniformis* and *B. mojavensis* and surfactin being mainly produced by *B. subtilis* (Sen 2010a). Isoforms of surfactin vary in length or branching of its hydroxy fatty acid moiety (Yakimov et al. 1995). A curious finding is that surfactin produced by *B. subtilis* ATCC 21332 under aerobiosis and anaerobiosis presents the same five isoforms but not in the same proportions (Zhang et al. 2007).

Besides surfactin, *B. subtilis* subsp. *spizizenii* ATCC6633 is able to produce mycosubtilin in microaerobic conditions (Guez et al. 2008). Mycosubtilin is a nonribosomal lipopeptide belonging to the iturin family, with known antifungal and surface-active properties. Iturins are composed by a cyclic heptapeptide bonded to a β-amino fatty acid, which chain can vary between C_{15} and C_{17} (Fig. 1). The main difference between different iturins, including mycosubtilin, is the α amino acid composition of the heptapeptide (Peypoux et al. 1986; Fickers et al. 2008; Hamdache et al. 2013). *B. subtilis* BBG100 is a genetically

modified strain of *B. subtilis* ATCC6633 in which the promoter responsible for the mycosubtilin synthetase operon was replaced by a constitutive promoter from *Staphylococcus aureus* plasmid pUB110. *B. subtilis* BBG100 is able to produce surfactin and mycosubtilin under microaerobiosis, achieving higher mycosubtilin but lower surfactin concentrations than *B. subtilis* ATCC6633 (Table 1; Guez et al. 2008).

The fermentative *B. subtilis* C9 (KCTC 87 01P) preferred oxygen-limiting condition for the production of BSF C9-BS (Kim et al. 1997). BSF production was increased when a 15-h period of microaerobiosis and anaerobiosis was imposed during the exponential phase in batch fermentation. BSF production started in early exponential phase and decreased during stationary phase. As expected, a shorter lag period was observed in aerobic conditions but biomass and BSF production rates were half and a third, respectively, than in oxygen-limiting condition. Since C9-BS, a lipopeptide-type BSF, is also an emulsifier, it leads to the formation of foam in bioreactors, which is aggravated by the presence of oxygen. Foam overflow causes loss of medium, leading to lower biomass and BSF production. C9-BS presents a CMC of 40 mM and is able to lower the surface tension of water from 72 to 29 mN/m. Activity is stable in wide ranges of temperature, pH, salinity (Table 3) and up to 10 mM of $CaCl_2$ concentration (Kim et al. 1997).

Bacillus amyloliquefaciens S499, a fermentative strain found in the rhizosphere of plants, such as tomato, was the first strain reported to produce metabolites from three known families of *Bacillus* BSFs (surfactin, iturins and fengycin) in anaerobic conditions (Nihorimbere et al. 2012). Fengycins are peptides composed by 10 α-amino acids, similar to surfactin, in which the decapeptide is linked to a β-hydroxy fatty acid from C_{16} to C_{19} (Hamdache et al. 2013). In anaerobic cultures, total BSF production rate was more than double than in aerobic cultures. The proportion between different BSF was similar in both conditions with surfactin being the most abundant (67%), followed by iturins (29%) and fengycin (4%) (Nihorimbere et al. 2012).

2.3 Pseudomonas

Pseudomonas includes the species *P. aeruginosa,* an opportunistic human pathogen. *P. aeruginosa* infections are often accompanied by formation of biofilms, e.g. in respiratory tract of cystic fibrosis patients (Worlitzsch et al. 2002; Van Alst et al. 2007). As such, it is considered to be a model organism for the study of formation, maintenance and dispersal (also known as detachment) of biofilms (De Kievit 2009). In *P. aeruginosa*, BSF production is involved in the regulation of biofilm development (Wang et al. 2014). Rhamnolipids produced by *P. aeruginosa* and other pseudomonads, are glycolipids composed of a single or dimer of β-hydroxy fatty acid glycosylated to a mono- or di-rhamnose (Fig. 1). The fatty acid chains can range from C_8 to C_{12} (Soberón–Chávez et al. 2005). Rhamnolipids have been found to deeply affect all the three stages of biofilm development. This is mainly due to their involvement in quorum-sensing mechanism, a cell-density-dependent type of cell-to-cell communication (Hassett et al. 2002). Rhamnolipids are involved in early biofilm microcolony formation (Pamp and Tolker-Nielsen 2007), while in later stages, they are fundamental in the maintenance of the biofilm architecture (De Kievit 2009). The typical architecture of single-species *P. aeruginosa* biofilms corresponds to several micro-colonies, which in mature biofilms form mushroom-like structures separated by open channels, attached to a surface. Fluids can circulate through biofilm channels, delivering nutrients and terminal electron acceptors, and removing metabolic products (Davey et al. 2003). Rhamnolipids are involved in the maintenance of open channels in mature biofilms, by affecting interactions between cells, or between cells and the interface or solid surface (Davey et al. 2003) and preventing competitors from adhering to the open areas of the channels (Espinosa-Urgel 2003). Rhamnolipids are also responsible for the formation of the cap in mushroom-like structures (Lequette and Greenberg 2005; Pamp and Tolker-Nielsen 2007) and contribute to the dispersion of cells by causing the formation of cavities in the center of the biofilm, from which cells detach (Schooling et al. 2004; Boles et al. 2005). Most studies of BSF production on Pseudomonads have been conducted in aerobic conditions. However, Yoon et al. (2002) reported that *P. aeruginosa* not only forms robust biofilms under anaerobiosis but also prefers anaerobiosis for biofilm development. Even biofilms produced in aerobic conditions have large anoxic zones, because oxygen diffusion is limited to the upper 50–90 μm (Walters et al. 2003), which points to the possibility of rhamnolipid production

under biofilm anaerobic conditions. In vitro anaerobic biofilms are thicker and more compact than aerobic counterparts (Yoon et al. 2002), which may indicate a deregulation of the formation of the mushroom-like structures and the maintenance of the fluid channels, which are regulated by the production of rhamnolipids. Nonetheless, well separated micro-colonies have been observed in vivo, although the techniques used were not suitable for the observation of mushroom-like structures (Worlitzsch et al. 2002). Therefore, the production rhamnolipids in anaerobic biofilms is still to be confirmed.

Anaerobic rhamnolipid production has been detected in *P. aeruginosa* PAO1 T, a NRB, related to its swarming ability in high agar (1.5–2.5%) medium (Nozawa et al. 2007). Swarming is a kind of flagellum-dependent motility on solid surfaces. In this case mutants lacking both flagellum and pili were still able to spread in high agar plates. In *P. aeruginosa,* rhamnolipid is required for swarming, since mutants lacking the *rhlA* gene, required for rhamnolipid production, display a non-swarming phenotype. Swarming activity was restored with insertion of the lacking gene or addition of bio- or synthetic surfactants to the medium. Interestingly, swarming in agar plates was enhanced in anaerobiosis with 8% CO_2 (Nozawa et al. 2007).

The formation of dense and voluminous foam, intrinsically related to the presence of oxygen, is a problem associated with aerobic fermentation for rhamnolipid production and several attempts of rhamnolipid production in denitrifying conditions have been made in order to circumvent foam production and oxygen limitation. *P. aeruginosa* ATCC 10145 in planktonic state presented an average rhamnolipid production rate of 2 $mg/g_{cell\ protein}{\cdot}h$ when cultured in denitrifying conditions, which corresponded to approximately 1/3 of the aerobic production. However, the production process could be optimized with higher cell concentrations, since little or no foam was formed and there was no oxygen limitation (Chayabutra et al. 2001). The immobilization of *P. aeruginosa* E03-40 in hollow-fiber bioreactors working under denitrifying conditions has also been applied as a strategy to reduce foaming (Pinzon et al. 2013). The BSF was produced for over 1500 h with a specific productivity of 17 $mg/g_{biomass}{\cdot}h$, similar to that of aerobic conditions. In an initial phase, the bioreactor presented microaerobic conditions and in a later phase, surface aeration was removed so that the conditions were as close to anaerobiosis as possible. However the mass transfer of the BSF through the membrane was affected by concentrations above the BSF CMC due to the formation of micelles and other aggregates (Pinzon et al. 2013). This method coupled with continuous extraction of rhamnolipids may prove to be an interesting alternative for industrial rhamnolipid, and possibly other BSFs, production.

Three BSF-producing hydrocarbonoclastic bacteria isolated from enrichment cultures of contaminated soil and crude oil were identified as *Pseudomonas* sp. BS2201 and *Pseudomonas* sp. BS2203 based on their biochemical characteristics (Grishchenkov et al. 2000). These are some of the first strains described to be able to produce BSF under both aerobic and anaerobic conditions using crude oil as sole carbon source (Grishchenkov et al. 2000).

Pseudomonas sp. ANBIOSURF-1 was isolated from enrichment cultures of sewage sludge and initially showed potential for MEOR strategies due to its ability to produce BSF in anaerobic environments (Albino and Nambi 2010). It should be noted that in pre-culture anaerobic conditions, sulfate was the only terminal electron acceptor added to the medium, while in anaerobic BSF production nitrate was also added. Further tests revealed that the BSF produced using coconut oil as substrate was a glycolipid, most likely a pure mono rhamnolipid with a CMC of 52 mg/L. Furthermore, the BSF presented better emulsifying ability when tested with chlorinated compounds than with petroleum hydrocarbons (Albino and Nambi 2010).

The genetic engineering of the facultative anaerobe *Pseudomonas stutzeri* DQ1 by the addition of the rhamnosyltransferase gene *rhlABRI* from the aerobe *P. aeruginosa* SQ6 resulted in a *P. stutzeri*, strain Rhl, able to reduce nitrate and effectively produce rhamnolipids in anaerobic conditions (Zhao et al. 2015c). Through the use of statistical modelling, key nutrients for rhamnolipids production were identified and medium composition was optimized leading to a BSF yield of 3.12 g/L (Zhao et al. 2014). Like for most facultative anaerobes, growth was faster and biomass and BSF yields were higher under aerobic conditions. BSF was produced almost in parallel with cell growth during exponential phase (24 h), reaching maximum concentration (3.10 g/L) during stationary phase (Zhao et al. 2014). Furthermore, thin layer chromatography and Fourier transform infrared spectroscopy

confirmed that the rhamnolipid produced by *P. stutzeri* Rhl was similar to that produced by the original carrier of the gene *rhlABRI,* with a CMC of 90 mg/L and highly active within a wide range of temperatures, salinities and pH (Table 3; Zhao et al. 2015c).

Two BSF-producing facultative anaerobic *Pseudomonas aeruginosa* strains, SG and WJ-1, were isolated from oil reservoirs (Zhao et al. 2015b). The BSF produced aerobically by the WJ-1 strain presented a CMC of 14 mg/L (Xia et al. 2012). While the rhamnolipid anaerobically produced by the *P. aeruginosa* SG presented a CMC of 80 mg/L and maintained its activity under different pH, temperature and pressure values (Table 3; Zhao et al. 2015d). *P. aeruginosa* SG itself was shown to produce BSF under wide ranges of temperatures, pH and salinities (Table 2; Zhao et al. 2015d). *P. aeruginosa* SG was used to test the effect of increasing the number of *rhlAB* genes, responsible for expression of rhamnosyltransferases, and the replacement of the original promoter for a stronger one, in rhamnolipid production under both aerobic and anaerobic conditions. Two modified strains were tested: *P. aeruginosa* PrhlAB corresponding to the transformed *P. aeruginosa* SG with the recombinant plasmid pBBRPrhlAB, which contains *rhlAB* genes with native promoter, and *P. aeruginosa* PoprAB containing the recombinant plasmid pBBRPoprAB carrying *rhlAB* genes and the strong promoter of *oprL* gene, responsible for the expression of peptidoglycan-associated lipoproteins (Zhao et al. 2015a). BSF production started at early exponential phase, peaking at the end of the stationary phase in all tested strains. Increasing the number of *rhlAB* gene copies resulted in a 1.47-fold increase of BSF produced by strain PrhlAB in comparison to the wild type, strain SG, while the cumulative effect of increasing the number of copies and adding a strong indigenous promotor led to a 3.30-fold increase, by strain PoprAB, under anaerobic conditions (Zhao et al. 2015a). These strategies have proven that overexpression of important genes associated with BSF production is possible even under oxygen-limiting condition. Thus the use of genetically modified anaerobic BSF producing bacteria may lead to improved BSF yields. Potentially making these bacteria more enticing to be used in biotechnological applications.

2.4 Anaerophaga

Two halo- and thermotolerant strict anaerobes able to produce BSF were isolated from oil contaminated sedimentary tanks in Germany. Both isolates have been initially classified as belonging to the *Bacteroides* genus (Denger and Schink 1995) but later assigned to the new genus *Anaerophaga* (Denger et al. 2002). One of the isolates was identified as *Anaerophaga thermohalophila* Fru22 (DSM 12881). The other isolate, identified as Glc12, presents very similar physiological and phenotypic characteristic to strain Fru22. These two isolates were strictly fermentative, not using oxygen, nitrate, sulfate, thiosulfate, sulfite, sulfur or fumarate as terminal electron acceptors. Production of BSF in anaerobic cultures was initiated in late exponential phase and continued throughout stationary phase. The produced BSFs were not identified but they did not alter the viscosity of culture media and were able to stabilize emulsions (Denger and Schink 1995). Fru22 BSF had a molecular weight <12 kDa, was stable at temperatures up to 70 °C during 20 min, presented optimal activity at pH 2 and was susceptible to proteinase K and trypsin, but not lipase. It was described as an oligopeptide, with attached fatty acids and may contain sugar residues (Denger et al. 2002). Both strains gather several favorable characteristics for applications: high temperature and salt tolerance, lack of formation of gases from fermentation, including CO_2, fast growth and production of a thermotolerant BSF (Denger and Schink 1995; Denger et al. 2002). However, a setback is that strain Fru22 was not able to grow on solid media (Denger et al. 2002).

2.5 Bretibacillus

Bretibacillus sp. BS2202 is a nitrate-reducing hydrocarbonoclastic bacteria isolated together with two *Pseudomonas* strains from oil and oil-contaminated sediments. As the other two isolates, it was able to metabolize petroleum hydrocarbons and produce BSF both under aerobic and anaerobic conditions. Albeit, the decrease of surface tension in anaerobic conditions was smaller than in aerobiosis (Grishchenkov et al. 2000).

2.6 Clostridium

One of the first anaerobic BSF producers identified was a *Clostridium pasteurianum* strain. This fermentative bacterium was able to produce a BSF under anaerobiosis using sucrose as a carbon source. The BSF was able to reduce the surface tension of the growth medium to 55 mN/m (Cooper et al. 1980).

2.7 Desulfovibrio

La Rivière (1955) showed for the first time that a bacterium, the SRB *Desulfovibrio desulfuricans* El Agheila Z, could produce BSF under anaerobic conditions. A decrease of surface tension of the medium of 21 mN/m in 7 days was reported. The author hypothesized that *D. desulfuricans* released surfactant to the medium not only through excretion but also by autolysis.

2.8 Geobacillus

Geobacillus pallidus H9 is a halo- and thermotolerant NRB, isolated from a Chinese oil reservoir (Wenjie et al. 2012). It was able to produce BSF under both aerobic and anaerobic conditions, although growth and BSF yields were higher in the former. BSF composition changed in relation to the oxygenation conditions. In BSFs produced under aerobic and anaerobic conditions, the fractions of glycosides were 50.3 and 53.8% (w/w surfactant), the lipids were 34.5 and 31.2% and the peptides represented 15.2 and 26.0%, respectively. This corresponded to slightly different CMC values for the BSF produced in aerobiosis (16 mg/L) and anaerobiosis (22 mg/L) and by a stronger reduction of medium surface tension in aerobic cultures (Table 1). BSF yield, cell surface lipophilicity and emulsification capacity were negatively affected by anaerobiosis. The maximum BSF yields were of 9.80 and 2.16 g/L, cell surface lipophilicity were 88.4 and 64% and emulsification indexes at 24 h were 90–95% and 10–35% for the BSF produced in the presence or in the absence of oxygen, respectively (Wenjie et al. 2012).

2.9 Rhodococcus

Species of *Rhodococcus* genus are known aerobic producers of intracellular BSF. *Rhodococcus ruber* Z25, a NRB isolated from an oil well in China was able to grow and produce BSF in anaerobic conditions, albeit presenting much lower biomass and BSF concentrations (0.11 and 0.53 g/L, respectively) than those achieved in aerobic cultures (1.46 and 12.95 g/L, respectively) (Zheng et al. 2012).

2.10 Thermoanaerobacter

Yen et al. (1991) demonstrated the production of an extracellular glycopeptide BSF by *Thermoanaerobacter pseudethanolicus* 39E, formerly *Clostridium thermohydrosulfuricum* 39E, under anaerobiosis. Isolated from hot springs, this extremophile is a strict anaerobe with very promising characteristics for use in biotechnological applications, since it is thermophilic, halotolerant and spore forming (Yen et al. 1991). This is one of the few SRB known to produce BSF in oxygen-limiting conditions.

A mixed culture, predominantly composed by a *Thermoanaerobacter* strain, isolated from heavy oil samples has been shown to produce BSF at reservoir conditions (70 °C, 15 g/L NaCl) and using molasses as substrate. Additionally this culture was able to grow under pressures of 0.8–14.2 MPa and production of methane was observed (Castorena-Cortés et al. 2012).

3 Effect of oxygen depletion on BSF production pathways

Very little is known about the regulation of BSF production under microaerobic or anaerobic conditions. It is assumed that metabolic pathways are identical to those operating in aerobic conditions (McInerney et al. 1990) and comprehensive descriptions of the genetics and biochemistry of bacterial BSF production can be found in specific reviews (Sullivan 1998; Soberón–Chávez et al. 2005; Das et al. 2008; Sen 2010b; Reis et al. 2011).

Two of the most common BSF produced under oxygen-limiting conditions are surfactin and rhamnolipids, by *Bacillus* and *Pseudomonas* species, respectively. In general, the biosynthesis of surfactin by *Bacillus* is thought to be non-ribossomal depending instead on the activity of peptide-synthetases. These multienzyme complexes catalyze the synthesis of peptides using directly precursor amino acids without any need for ribosomal protein synthesis. Biosynthesis

of surfactin ends when the molecule becomes cyclic or is released by the complex. Operon *srf* contains the genes responsible for the regulation of surfactin synthesis and encodes for some of the enzymes subunits (Das et al. 2008; Sen 2010a). Rhamnolipids produced by *Pseudomonas* species are mainly regulated by the *rhl* quorum-sensing system. The biosynthesis of rhamnolipids is the result of the junction of two pathways, the biosynthesis of dTDP-L-rhamnose and the biosynthesis of the fatty acid moiety, by two consecutive rhamnosyltransfer reactions. Each reaction is catalyzed by a specific rhamnosyltransferase. The first is encoded by the genes *rhl A* and *B* and the second by the gene *rhlC* (Das et al. 2008; Reis et al. 2011).

Some studies addressed specifically the influence of oxygen depletion on BSF production. In the previously mentioned *P. stutzeri* Rhl, the ability to produce BSF was added to a facultative anaerobic non-BSF producing *P. stutzeri* strain (Zhao et al. 2015c). This was interpreted as evidence of rhamnolipid production in Pseudomonads not being directly dependent on oxygen, even if oxygen availability is likely to influence other metabolic pathways, namely those related with organic carbon consumption (Chayabutra et al. 2001). Furthermore, it should be noted that the original strains, *P. aeruginosa* SQ6 and *P. stutzeri* DQ1, are close relatives and may share regulatory mechanisms (Zhao et al. 2015c).

Even if BSF production is not directly dependent on oxygen, there is evidence that the absence of oxygen can indirectly affect the metabolic pathways involved in the production of BSF. In several cases BSFs produced under aerobic and anaerobic conditions differ in the relative proportion of different components (Zhang et al. 2007; Wenjie et al. 2012). It has been hypothesized that under anaerobic conditions, substrate preferences change due to the redox potential of the biochemical reactions. This, coupled with the usage of different electron acceptors, may lead to changes in the metabolic pathways involved in the production of BSF.

In a study conducted during a spaceflight mission, a transformed *P. aeruginosa* PAO1 resistant to gentamicin adopted anaerobic growth (Crabbé et al. 2011). The culture was kept inside the spacecraft, under aerobic conditions, but exposed to microgravity and low fluid shear, resulting in a less efficient oxygenation of the liquid culture medium than in the control, incubated on Earth. Most genes upregulated in relation to the control were related with anaerobic growth, especially denitrification. Additionally, gene *rhlA*, involved in the synthesis of rhamnolipids, was among the most upregulated genes. However, gene *rhlI*, responsible for the *N*-butanoyl-L-homoserine lactone synthase and also involved in rhamnolipid production, was down regulated (Crabbé et al. 2011). Furthermore, biofilm formation by *P. aeruginosa* was observed during the spaceflight, although the presence of rhamnolipids was not directly confirmed (Kim et al. 2013). The combination of transcriptomic and metabolomics approaches could shed some light on the regulation of BSF synthesis in sub-oxic conditions.

Generally, the isolates presented on Table 1 present higher BSF yields under aerobiosis than in oxygen-limiting conditions. Primary metabolic pathways that are not dependent on O_2 produce less energy than their aerobic counterparts (Nelson and Cox 2005). Since BSFs are usually secondary metabolites (Davis et al. 1999), it is possible that their production is reduced under oxygen-limiting conditions considering that the available energy is preferentially used in vital metabolic pathways instead of BSF production. On the other hand, less energy results in lower cell growth which implicates less BSF production when BSF production is growth-dependent (further discussed later in this review). In cases where BSF yields are higher under oxygen-limiting conditions (Lin et al. 1994a; Kim et al. 1997; Gogotov and Miroshnikov 2009; Nihorimbere et al. 2012) it is possible that non-vital pathways that were being preferentially used in aerobic conditions are now less used and the energy redirected towards BSF production.

Because changes in BSFs composition affect their properties, such as the CMC and emulsification index (Wenjie et al. 2012), it is important to understand, from the molecular perspective, how and why these shifts occur, in order to design efficient cultivation conditions to achieve specific production outcomes. Unfortunately, this information is still very scarce in scientific literature.

4 Growth conditions and optimization of media for BSF production under oxygen-limiting conditions

Bacterial growth is affected by several factors, among which the composition of the culture medium is

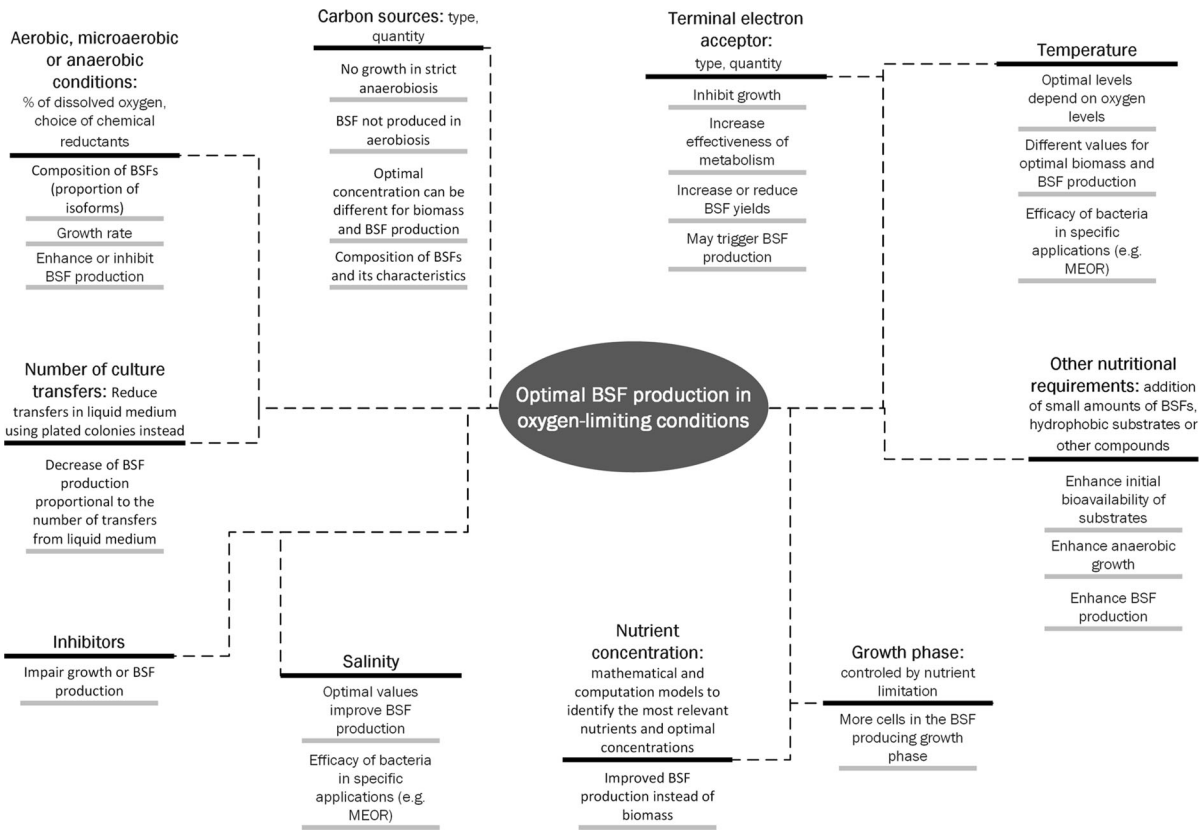

Fig. 2 Main parameters that can affect BSF production in oxygen-limiting conditions and their possible effects

extremely significant. The optimal growth conditions for BSF production should achieve the highest yields and the best product quality. A scheme summarizing the main effects each factor can have on BSF production in oxygen-limiting conditions is presented in Fig. 2.

4.1 Aerobic, microaerobic or anaerobic conditions

Some bacteria produce higher amounts of BSF under low oxygen availability. Strict anaerobiosis can be achieved in several ways, but most often by addition of a chemical reductant to the culture medium, most frequently sodium sulfide (Na_2S), cysteine-HCl or ascorbate (Plugge 2005; Widdel 2010). The choice depends on several parameters such as the degree of reduction of medium intended, the possibility of being used as a carbon source, the formation of precipitates with other compounds or bacterial toxicity.

A concentration of 2.0 g/L of Na_2S was reported as the most adequate for anaerobic production of BSF by *B. licheniformis* VKM B-511 (Gogotov and Miroshnikov 2009). On the other hand, the use of a solution of cysteine-HCl and $Na_2S{\cdot}9H_20$ as chemical reducing agents showed to negatively affect growth and BSF production in *B. mojavensis* JF-2, because sulfide inhibits the activity of key enzymes in some nitrate-reducing bacteria (Javaheri et al. 1985). Production of lichenysin B by *B. mojavensis* JF-2 was the highest with 30% dissolved oxygen in media with 2% (w/v) NaCl, although biomass production is higher with 85% oxygen saturation (Lin et al. 1994a). Other studies showed the production of acceptable levels of BSF in *B. mojavensis* JF-2 under anaerobiosis (Javaheri et al. 1985; Marsh et al. 1995). Hence, it was proposed that BSF production by *B. mojavensis* JF-2 may be more dependent on growth rate than on O_2 availability, although the growth rate is influenced by dissolved oxygen and NaCl concentrations. As such, slower

growth would lead to a longer exponential phase, when the BSF is produced and released, and a later stationary phase, when uptake of BSF occurs (Lin et al. 1994a). Other bacterial species have maximum BSF production yields under anaerobic and microaerobic conditions. That is the case of *B. licheniformis* VKM B-511 (Gogotov and Miroshnikov 2009) and *B. subtilis* C9 (Kim et al. 1997). *B. amyloliquefaciens* S499 also presents a higher BSF production rate in anaerobiosis than aerobiosis (Nihorimbere et al. 2012).

A bioreactor study with *B. subtilis* ATCC 21332 demonstrated that surfactin production is affected by aeration and agitation. Optimized surfactin production conditions, 1.5 vvm and 300 rpm, resulted in an almost complete exhaustion of O_2 during exponential growth, with surfactin concentration reaching 6.45 g/L and surfactin production yields of 161 mg/$g_{glucose}$ (Yeh et al. 2006).

Oxygenation also affects the composition of the BSF produced. A study addressed the regulating effect of oxygen in the production of mycosubtilin, and to a smaller extent surfactin in *B. subtilis* ATCC6633 and *B. subtilis* BBG100 (Guez et al. 2008). An increase of 25-fold in mycosubtilin production was observed in *B. subtilis* ATCC6633 cultures with the lowest oxygen rate transfer tested, 7 mmol O_2/L·h, in comparison to the highest, 20 mmol O_2/L·h. This resulted in an increase in mycosubtilin concentration of 2.8–45.4 mg/L and mycosubtilin specific productivity from 0.001 mg/$g_{biomass}$·h to 0.025 mg/$g_{biomass}$·h, from the highest tested oxygen rate transfer to the lowest. In *B. subtilis* BBG100 cultures both mycosubtilin concentration (67.9–82.2 mg/L) and specific productivity (0.033–0.037 mg/$g_{biomass}$·h), remained similar independently of the oxygen rate transfer. Mycosubtilin and surfactin metabolisms share an identical cofactor, which can limit surfactin production when mycosubtilin is over produced. The native promoter of mycosubtilin synthetase operon was shown to be at least in part, responsible for the oxygen regulation of mycosubtilin production. *B. subtilis* ATCC6633, which contains the native promoter, is affected by oxygen rate transfer and *B. subtilis* BBG100, which contains an exogenous promoter, is barely affected. Additionally, differences in the production of BSF isoforms were reported, with an increase in the percentage of mycosubtilin with C_{17} fatty acid chains and a decrease in the C_{16} isoform with increasingly microaerobic conditions. This indicates that oxygen may also be involved in regulating the production of lipopeptides (Guez et al. 2008). Likewise, surfactin produced under aerobic and anaerobic conditions can have different percentage of isoforms. HPLC spectra of surfactin produced by *B. subtilis* ATCC 21332 show the same five isoforms present for both BSFs, but the mass concentration of each isoform varies. When the recovered surfactins were tested, induction of gas hydrate formation was significantly more effective for surfactins produced under anaerobic conditions (Zhang et al. 2007). Therefore, differences in the proportion of isoforms present affect BSF activity.

In *G. pallidus* H9, substrate uptake varies between aerobic and anaerobic conditions. This, is believed to cause differences in the constitution of the BSF produced, which resulted in different values of CMC, cell surface lipophilicity and emulsification (Wenjie et al. 2012).

The optimal conditions for growth and spreading of *P. aeruginosa* PAO1 T, in which swarming is rhamnolipid-dependent, are anaerobic conditions supplemented with 8% CO_2 (Nozawa et al. 2007).

It should be noted that some bacteria that produce BSF under aerobiosis may completely fail to do it under oxygen deprivation. Two *P. aeruginosa* strains that produced BSFs with good emulsifying activity in aerobic conditions, were able to grow in the exact same medium under anaerobiosis but neither a decrease of surface tension nor emulsification were detected (Gudiña et al. 2012). The same was observed in another study with *P. aeruginosa* CVCM 411 (De Rienzo et al. 2014). In other cases, the time of production under anaerobic conditions could significantly increase. Additionally, BSF and biomass yields are also usually lower under anaerobic conditions. As an example, production of BSF both in PPGA medium and MSM with crude oil by three strains, two *Pseudomonas* sp. and one *Brevibacillus* sp., took 10 days in aerobic conditions and 50 days in anaerobiosis (Grishchenkov et al. 2000). Moreover even with the extra reactional time, usually surface tensions of anaerobic media at the end of fermentation are never as lower as values obtained under aerobic conditions (Grishchenkov et al. 2000), which could be a consequence of reduced BSF production.

In general, oxygen availability can affect BSF production, rate and yield as well as BSF composition and isoform percentage, and consequently, overall BSF properties. Therefore, oxygenation is a critical

parameter in BSF production with considerable impact in the efficiency of the process and on quantity and quality of BSF produced.

4.2 Terminal electron acceptors

The most common terminal electron acceptors used by bacteria under anaerobic conditions are sulfate (SO_4^{2-}) and nitrate (NO_3^-). However, some other less common terminal electron acceptors, such as manganese (Mn^{4+}), nitrite (NO_2^-), sulfur (S^0), sulfite (SO_3^{2-}), carbonate (CO_3^{2-}), iron (Fe^{3+}), carbon dioxide (CO_2) or fumarate, may additionally be used (Madigan et al. 2014). All BSF producers capable of operating anaerobic respiration described in this review are either SRB, NRB or use organic compounds as terminal electron acceptors (Table 2).

Pseudomonas aeruginosa growing under anaerobic conditions preferably uses nitrate as terminal electron acceptor (Vander Wauven et al. 1984), although high concentrations of nitrate may inhibit growth (Chayabutra and Ju 2000; Zhao et al. 2016b). It can also use nitrite or arginine as alternative acceptors (Vander Wauven et al. 1984). Surfactin batch production by *B. subtilis* ATCC 21332 also relies on nitrate reduction (Davis et al. 1999). Under anaerobiosis nitrate was used as the preferred terminal electron acceptor and once depleted, nitrite was used. This transition actually corresponded to an enhancement in BSF production. When nitrate was not limiting, nitrite is not used, resulting in lower BSF yields (Davis et al. 1999). In this case, it appears that the increase in BSF production was not directly triggered by oxygen depletion *per se* but rather by the use of nitrate, which the authors believe to be involved in signaling the start of the secondary metabolism related to the production of BSF by the bacteria (Davis et al. 1999). In an anaerobic study aiming to access the impact of addition of nitrate in the microbial community, oil souring and potential for MEOR application, BSF production was detected. This consisted in water flooding experiments in sand packed columns inoculated with a microbial consortium isolated from a Brazilian offshore oil (da Silva et al. 2014). The produced BSF, which was detected by a decrease in interfacial tension, considered to be partly responsible for the increase of 4.3% (v/v) of the initial oil recovered when compared to the culture without added nitrate (da Silva et al. 2014).

4.3 Carbon sources

Not all carbon sources used by a facultative anaerobe in aerobic conditions can be used during anaerobic growth. This happens because the metabolic pathways of some substrates require oxygen to be functional. As an example, the metabolization of hexadecane by *P. aeruginosa* ATCC 10145 for the production of rhamnolipids, was only possible in the presence of oxygen (Chayabutra and Ju 2000; Chayabutra et al. 2001). When alternative carbon sources were tested (palmitic acid, stearic acid, oleic acid, linoleic acid, glycerol, vegetable oil and glucose) anaerobic production of rhamnolipids was only observed in the cultures with palmitic or stearic acids. Palmitic acid rendered the best production which, albeit, in aerobic conditions, it corresponded at the best, to 82% of the productivity achieved with hexadecane (Chayabutra et al. 2001). Therefore, the selection of the carbon source for anaerobic cultures should take into account not only the capacity of bacteria to metabolize it in absence of O_2 but also the BSF production efficiency of the culture.

While in most anaerobic studies either sugars or petroleum hydrocarbons are used as substrates, other lower cost substrates, such as vegetable oils, can be used. Anaerobic rhamnolipid production was documented in *Pseudomonas* sp. ANBIOSURF-1 growing on coconut oil (Albino and Nambi 2010). Glycerol, a byproduct of biodiesel and soap production, was also identified as the carbon source that achieved lower medium surface tension values in anaerobic *P. aeruginosa* SG cultures. Other low cost substrates that led to a less effective production of anaerobic BSF by *P. aeruginosa* SG were sunflower oil and soybean oil (Zhao et al. 2015d, 2016b).

Substrate concentration can also influence biomass and metabolite production, including BSF. Tests performed in a mixed culture composed mainly of *Thermoanaerobacter* sp. revealed that maximum biomass and CO_2 yields were obtained at concentrations of glucose <1.68 g/L. However, BSF production was only observed at initial substrate concentration above 5.8 g/L (Castorena-Cortés et al. 2012). In a study addressing the production of rhamnolipids by *P. aeruginosa* SG the best production yield was obtained at glycerol concentration of 72 g/L, and production yield was positively correlated with the concentration of carbon source within

the range of 12 g/L up to 72 g/L. These studies indicate that high yields of BSFs require considerable concentrations of carbon sources (Zhao et al. 2016b).

Substrate type affects the properties of the produced BSF. Studies on the effect of carbon source in *B. subtilis* C9 showed that different carbon sources affected BSF production, surface tension of the medium and emulsification activity of the broth. Out of several media with different carbon sources, emulsification of crude oil was only observed in glucose broth, which was also the medium showing lowest surface tension and highest BSF yield and production rate. Authors noted a direct correlation between BSF production, bacterial growth and glucose uptake. However, concentrations of glucose higher than 40 g/L had little effect on BSF production (Kim et al. 1997).

Petroleum hydrocarbons have also been used as substrates by BSF producing bacteria. Five *B. subtilis* strains were able to grow and produce BSF in anaerobic conditions using a mixture of long-chained alkanes (C_{20}–C_{30}) as sole carbon source (Gudiña et al. 2012). Gas chromatography analysis showed that most of the tested strains were able to degrade *n*-alkanes with C_{27} or higher and some were also able to degrade n-alkanes between C_{18} and C_{20}. Furthermore, the addition of *n*-hexadecane to a rich medium did not affect anaerobic BSF production (Gudiña et al. 2012). *G. pallidus* H9 was able to grow with crude oil as carbon source and produce BSF both under aerobic and anaerobic conditions (Wenjie et al. 2012). In aerobic cultures, medium- and long-chained hydrocarbons (C_{23}–C_{43}) were used preferentially, with hydrocarbons C_{41}–C_{43}, which were initially present in smaller concentrations, being completely metabolized. In anaerobic cultures, an overall preference for small- (C_8–C_{11}) and long-chained hydrocarbons (C_{32}–C_{43}) was observed and the hydrocarbon fraction C_{39}–C_{43} was totally degraded. Consequently, differences in the BSF composition were observed. BSF with different CMC, 16 and 22 mg/L, and emulsification indexes of 90–95% and 10–35%, were obtained in aerobic and anaerobic conditions, respectively. The anaerobically-produced BSF presented higher glycosidic and peptidic fractions and lower lipidic fractions than the BSF produced in aerobic conditions (Wenjie et al. 2012).

4.4 Other nutritional requirements

In addition to bulk carbon sources, some bacteria have other nutritional requirements for growth or production of BSF in anaerobiosis or microaerobiosis (Table 2). *B. mojavensis* JF-2 requires yeast extract to grow anaerobically in mineral modified medium E (Javaheri et al. 1985). Additionally it also requires deoxyribonucleosides or DNA to grow under strict anaerobic conditions. No such requirements are observed in aerobic growth of this strain. This is thought to be due to the lack of ribonucleotide reductases able to operate in anaerobiosis, which are needed to convert ribonucleotides into deoxyribonucleotides (Folmsbee et al. 2004). Addition of tryptone, proteose peptone or neopeptone has also been reported to be critical for anaerobic growth and BSF production in several bacteria (Castorena-Cortés et al. 2012).

Small amounts of BSF may be initially added to the culture medium to enhance solubility and bioavailability of the substrate and shorten the adaptation time of bacteria. However, some bacteria may also metabolize the added BSF which will actually provide a supplementary carbon source (Chayabutra et al. 2001). In the case of highly soluble carbon sources, the addition of small amounts of hydrophobic substrates, such as hexadecane, can serve as an additional stimulation and improve BSF production (Denger and Schink 1995).

Ions, particularly PO_4^{3-}, SO_4^{2-}, and Mg^{2+}, have been found to positively affect anaerobic rhamnolipid production by *P. aeruginosa* SG. Phosphate concentration in particular was found to be linked to BSF production yield (Zhao et al. 2016b). This anion is required for the formation of thymidine diphosphate (dTDP), which is essential to one of the rhamnolipids' precursors, dTDP-L-rhamnose (Reis et al. 2011). SO_4^{2-}, and Mg^{2+} are thought to be cofactors in enzymes involved in the rhamnolipid production pathways (Zhao et al. 2016b).

4.5 Nutrient concentration

The proportion in which particular supplements are provided also affect anaerobic or microaerobic BSF production. C/N ratios affect bacterial growth and BSF production and can be as important as the nature of the carbon or nitrogen sources. In a comprehensive study

of the optimal BSF production condition of *B. licheniformis* VKM B-511 optimal production yields were achieved with a 1:24 ratio (Gogotov and Miroshnikov 2009). In a similar study, the optimal C/N ratio for rhamnolipid production by *P. aeruginosa* SG was estimated as 1:16 (Zhao et al. 2016b).

A recent approach to media optimization used mathematical and computational models to provide predictions regarding cell growth and metabolites yields. This approach has been used for optimization of medium for anaerobic production of rhamnolipids by *P. stutzeri* Rhl (Zhao et al. 2014). In this case the most significant variables in the media composition were identified as glycerol, yeast extract and KH_2PO_4. Further analysis was performed to determine optimal concentrations. The fermentations used for the validation of the model obtained an average BSF yield (3.12 g/L) very close to the maximum predicted (3.26 g/L) and the model was, therefore, considered to be accurate and reliable. The optimized medium almost doubled the BSF yield when compared to the original medium (1.68 g/L) (Zhao et al. 2014). In a similar study with *P. aeruginosa* SG, by the same group, glycerol, $NaNO_3$ and phosphate were identified as the significant variables in anaerobic production of rhamnolipid. An increase of 1.71-fold was observed when optimal medium was used instead of the initial one. Also in this case the estimated BSF yield (632.3 mg/L) obtained using optimal medium was close to the experimental one (650.1 mg/L) (Zhao et al. 2016b). Therefore the application of mathematical and computational models to the optimization of BSF production in anaerobiosis can be a powerful tool, especially advantageous in reducing costs in trial and error experiments in medium formulation. However it should be employed with a critical frame of mind and model validation should always be performed.

4.6 Inhibitors

Inhibition of anaerobic BSF production by particular compounds has been reported (Table 2). Crude oil was found to decrease biomass, BSF and polymer production on *B. licheniformis* BNP29 (Yakimov et al. 1997). This was a surprising observation since the strain was isolated from an oil reservoir and the presence of hydrocarbons or compounds with low bioavailability is usually related with increased BSF production.

In a study aiming to optimize culture media for anaerobic BSF production, organic sources of nitrogen such a yeast extract and peptone, had an inhibitory effect in anaerobic rhamnolipid production by *P. aeruginosa* SG (Zhao et al. 2016b). It is thought that since these sources can also be used as carbon sources they may not be as conductive to BSF production as nutrient-limiting conditions, which are easier to achieve using nitrate as nitrogen source (Zhao et al. 2016b). In the same study hydrophobic substrates were found to have a negative effect in the production of BSFs (Zhao et al. 2016b).

Sulfide is also known to directly, yet reversibly, inhibit BSF production (Zhao et al. 2015b, 2016a). As previously mentioned, sodium sulfide can be added as a chemical reductant to anaerobic media, however another source of sulfide that can affect bacteria in natural environments is hydrogen sulfide produced by SRB. *P. stutzeri* Rhl is tolerant to initial sulfide concentrations up to 33.3 mg/L, at higher concentrations sulfide removal decreases drastically and almost no BSF is produced (Zhao et al. 2016a). *P. aeruginosa* strains SG and WJ-1 are even less tolerant, with growth and BSF production being inhibited at sulfide concentrations above 10 mg/L, and unable to remove sulfide from medium (Zhao et al. 2015b). Increasing the volume of inoculum of BSF-producing bacteria attenuated the inhibitory effect of sulfide (Zhao et al. 2015b). Such a decrease in BSF production is thought to be the result of cell growth inhibition (Zhao et al. 2015b). Sulfide inhibition of anaerobic BSF production can be of particular importance to the success of MEOR strategies, as is discussed in a latter section.

4.7 Salinity

BSF production in marine bacteria is widely documented in scientific literature and anaerobic halotolerant BSF-producing bacteria are of particular interest in MEOR strategies, since oil reservoirs often present high salinity and low oxygen concentrations. Salinity concentrations tolerated by the different known BSF producing-bacteria under oxygen-limiting conditions and optimal values for BSF production are presented in Table 2. The table shows that known microaerobic and anaerobic BSF producers' salinity requirements range from zero to 150 g/L NaCl. In addition, the majority of the most halotolerant bacteria listed were previously isolated from crude oil (Yakimov et al.

1997; Gudiña et al. 2012). Only few studies have assessed the isolates optimal salinity concentration for BSF production. In all cases listed optimal salinity concentration for BSF production coincides with the optimal salinity for growth (Yakimov et al. 1995; Gogotov and Miroshnikov 2009). Since salinity is known to affect BSF yields (Marsh et al. 1995; Yakimov et al. 1995; Gogotov and Miroshnikov 2009), it is therefore an important parameter for optimization of BSF production.

4.8 Temperature

Optimal growth temperature of BSF-producing bacteria (Table 2) can differ depending on oxygenation. In *G. pallidus* H9 growth occurred between 40 and 80 °C, but optimal growth temperature was 65 °C under aerobic conditions and 70 °C under anaerobiosis (Wenjie et al. 2012). However, little is known on the direct effect of temperature in anaerobic BSF production (Castorena-Cortés et al. 2012; Gudiña et al. 2012; Wenjie et al. 2012). Yakimov et al. (1995) observed that *B. licheniformis* BAS50 lichenysin A and biomass yields in anaerobic cultures were affected by temperature. The highest production was achieved at 40–45 °C (Yakimov et al. 1995). Likewise, *B. licheniformis* VKM B-511 was able to grow in a range of temperatures from 20 to 40 °C. BSF yield was the highest at 30 °C even though maximum growth rate was achieved at 40 °C (Gogotov and Miroshnikov 2009). This demonstrates that optimal growth temperature and optimal temperature for anaerobic BSF production are not always the same.

Determination of optimal anaerobic growth and BSF production temperatures are important in industrial production of BSFs as well as in in situ bioremediation and MEOR strategies. In the first case to improve production. In the latter, because some of the targeted environments for application of MEOR or bioremediation strategies can present extreme temperatures, such as high-temperature oil reservoirs (Castorena-Cortés et al. 2012) or sediments in cold regions.

4.9 Growth phase

In most reports of BSF-producing bacteria under oxygen-limiting conditions, BSF production is dependent on growth phase. In all strains in which BSF production under oxygen-limiting conditions was studied alongside the growth curve, BSF production started during the exponential phase. Probably, indicating that the produced BSFs are secondary metabolites. In some cases BSF production peaked during the exponential phase and its presence is reduced during stationary phase (Javaheri et al. 1985; Kim et al. 1997; Yakimov et al. 1997; Wenjie et al. 2012) while in others, BSF production reached its maximum during stationary phase (Denger and Schink 1995; Davis et al. 1999; Chayabutra et al. 2001; Zhao et al. 2015c). Production of rhamnolipids by *P. aeruginosa* happens mainly in stationary phase and is dependent on cell density. A way of controlling and limiting *P. aeruginosa* growth, and subsequently the BSF production, is to impose nutrient limitation. In aerobic conditions, nitrogen is commonly the chosen limiting nutrient. However, under denitrifying conditions nitrogen is required as terminal electron acceptor and therefore cannot be in limited concentrations in the medium. A study designed to identify an alternative limiting nutrient to be used in BSF production by *P. aeruginosa* ATCC 10145 under anaerobic conditions revealed that Ca and Fe had no effect, Mg had modest effects and S limitation was comparable to N limitation (Chayabutra et al. 2001). In an anaerobic batch culture containing nitrate it was observed that *P. aeruginosa* SG started immediately consuming nitrate which led to a quick increase of biomass and the start of rhamnolipid production. However, once nitrate reached limiting concentrations and was eventually depleted the formation rate of the BSF increased (Zhao et al. 2015d, 2016b). BSF production by three strains of *Pseudomonas* was enhanced when grown in phosphate-limiting media with protease peptone and glucose, in comparison to growth in a minimal salts medium with crude oil (Grishchenkov et al. 2000). Limiting P availability by adding phosphorous-free medium to an anaerobic bioreactor with *P. aeruginosa*, substantially increased rhamnolipid production (Pinzon et al. 2013). This strategy has also been successfully used in anaerobic surfactin production by *B. subtilis* ATCC 21332. BSF yield in nitrate-limited medium under anaerobic conditions was more than threefold higher than in N-limited aerobic conditions (Davis et al. 1999). Therefore, nutrient limitation can be an important tool for controlling bacterial growth and increase BSF production in oxygen-limiting industrial conditions.

4.10 Number of culture transfers

Some strains seem to have a limit to the number of possible sub-cultivations. Javaheri et al. (1985) tested the capacity of *B. mojavensis* JF-2 to produce BSF after several generations, under aerobic and anaerobic conditions. They showed that for both conditions there was an increase of minimum surface tension with increasing numbers of transfers between liquid media (Javaheri et al. 1985). A latter study demonstrated the importance of using fresh inocula with as few serial transfers in liquid medium as possible. With a fresh inoculum of *B. mojavensis* JF-2, up to 24% of residual oil was recovered from a core displacement experience while an inoculum transferred 15 times was only able to recover <3% (Marsh et al. 1995). This loss of productive capacity was accompanied by other phenotypic changes. Normal producers presented volcano-shaped colonies in solid medium and low-activity producers formed round colonies (Javaheri et al. 1985). Plasmids were not detected in normal producers and therefore, the loss of BSF productive capacity could not be attributed to a loss of plasmids (Javaheri et al. 1985). The use of plated colonies as inoculant, in order to reduce the number of liquid media transfers may minimize this problem. Some BSF producers are stimulated by the presence of hydrophobic compounds in the medium (Denger and Schink 1995; Gudiña et al. 2012) and therefore, it is possible that the loss of productive activity is related with the release of selective pressure along transfers.

5 Applications of BSFs

There is an ever increasing number of applications for BSFs related with industry, health and environment. Given the scope of this review only applications likely to occur under anaerobic or low oxygen conditions will be addressed.

5.1 Bioremediation

Bioremediation refers to the removal of a contaminant from a contaminated environment through the mobilization of living organisms and acceleration of the natural biodegradation process. These strategies can be divided in two major groups: bioaugmentation, in which specialized organisms are added to the environment to metabolize the contaminants, and biostimulation, in which nutrients are added to enhance the degradation of the contaminant by endogenous bacteria (Megharaj et al. 2011).

The addition or production of BSFs often bring benefits to bioremediation strategies, especially when hydrophobic pollutants are involved, but that is not always the case (Bregnard et al. 1998). Biosurfactant addition in some cases may delay the bioremediation process because it is used as a carbon source, thus competing with the pollutant, or for being toxic to the degrading bacteria (Bregnard et al. 1998). For optimal results, BSF-producers for bioaugmentation strategies should be chosen not only in function of their ability to grow under the prevailing environmental conditions, but also considering the properties of the BSF and the effects on autochthonous bacterial community.

Anaerobic sludge reactors used in domestic and industrial wastewater treatment are often spiked with surfactants to enhance the biodegradation of certain compounds (Yeh et al. 1998; Chang et al. 2005). However due to environmental concerns, several studies have attempted replacing chemical surfactants with BSFs (Nakhla et al. 2003; Damasceno et al. 2014; Huang et al. 2015). Similarly, studies of in situ bioremediation in river sediments of several xenobiotics, such as decabromodiphenyl ether (Huang et al. 2014), tetrabromobisphenol-A (Chang et al. 2012) and tetrachlorobisphenol-A (Yuan et al. 2011), were conducted under anaerobic conditions and achieved promising results with the addition of BSFs, namely surfactin and rhamnolipids. In both strategies, it could be interesting to add directly BSF-producing bacteria which in some cases may also be able to degrade the pollutants and would reduce the process time and economic resources by elimination of extra steps in BSF production and extraction.

Removal of heavy metals from contaminated soil is another possible application of anaerobic BSF-producing bacteria. A previous review discussed the use of BSFs in such technologies, noting that their use has advantages over the use of chemical surfactants as well as the existence of several studies with success (Olaniran et al. 2013).

Bioremediation of petroleum hydrocarbons from natural environments represents one of the most promising applications of anaerobically-produced BSFs. It is often considered that hydrocarbonoclastic bacteria involved in these strategies benefit from the

presence of surfactants that enhance the hydrocarbons bioavailability (Swannell et al. 1996). Due to biodegradability and toxicity issues, BSF are often preferred to chemical dispersants (Bregnard et al. 1998; Mohan et al. 2006). As such, ideally the hydrocarbonoclastic bacteria are BSF-producers or alternatively, in strategies of bioaugmentation, BSF-producers are added together with the hydrocarbon degraders. While less frequent than the latter, some of most promising BSF-producing hydrocarbonoclastic bacteria to be used under anaerobic conditions include *Pseudomonas* sp. BS2201, *Pseudomonas* sp. BS2203 and *Bretibacillus* sp. BS2202 (Grishchenkov et al. 2000), *G. pallidus* H9 (Wenjie et al. 2012), *Rhodococcus ruber* Z25 (Zheng et al. 2012) and several *B. subtilis* (Gudiña et al. 2012). While these bioremediation strategies are usually applied under aerobic conditions, in some cases they are required in anaerobic environments, such as aquatic environments or anaerobic sediment layers. In a study of in situ bioremediation of an aquifer contaminated with diesel fuel under denitrifying conditions, (Bregnard et al. 1998), the addition of rhamnolipids at concentrations above their CMC, delayed hydrocarbon degradation because the BSF was used as carbon source by indigenous bacteria. The addition of rhamnolipids also led to an increase in microbial biomass that could potentially clog the pores in soil and hinder the bioremediation strategy. A synthetic surfactant, Triton X-100, was tested as well but also performed poorly by being toxic to biodegrading bacteria. In situ anaerobic production of the BSF was proposed as an alternative to overcome the competition with diesel fuel as a carbon source (Bregnard et al. 1998).

When the compatibility of lichenysin B produced ex situ by *B. mojavensis* JF-2 for anaerobic hydrocarbon bioremediation, was tested under methanogenic, nitrate- and sulfate-reducing conditions (Jennings 2005), overall biodegradation of toluene was not influenced by the BSF, either added or locally produced. However under specific conditions toluene biodegradation was affected. Under methanogenic conditions, toluene biodegradation was inhibited by BSF above CMC levels, while under sulfate-reducing conditions, degradation was stimulated with concentrations of BSF above the CMC and inhibited at concentrations below 1/4 CMC. Hexadecane degradation was observed in all the anaerobic conditions in the presence of BSF at levels above the CMC. However, no difference was noted regarding degradation levels between addition of pre-purified BSF and in situ production. No clear trend was observed on the degradation of naphthalene (Jennings and Tanner 2004; Jennings 2005). These results alert to the fact that a seemingly promising bacteria for bioremediation may only perform as wished under very specific conditions and for particular pollutants. This may help justify why, while there are several works concerning application of ex situ-produced BSFs and some laboratory scale studies about in situ BSF production, there is a lack of studies regarding the production of BSF in in situ during bioremediation strategies in oxygen-limited environments.

5.2 Microbial enhanced oil recovery (MEOR)

MEOR involves biostimulation or bioaugmentation strategies to promote favorable metabolism in bacteria that result in higher percentages of oil recovered from an oil well. Metabolic activities of interest include degradation of paraffin, inhibition of SRBs or production of BSFs, emulsifiers or polymers, among others (Sen 2008).

The use of surfactants in enhanced oil recovery (EOR) strategies is of particular interest due to their ability to lower interfacial surface tension between the hydrocarbon and the aqueous phases and rock present in oil wells. In normal conditions, the surface tension between these phases is high enough to trap hydrocarbons in the more porous strata, e.g. those including limestone, adjacent to oil wells. Addition of surfactants will therefore lower the surface tension, attenuating capillary forces, and allow for the displacement of oil from the pores by water ultimately resulting in increased oil production rates from oil wells (Sen 2008). However, for these approaches to work, surfactant or BSF concentration inside the reservoir must be well above the CMC levels (Sabatini et al. 1998; Youssef et al. 2007). In cases where surfactants are used considerable amounts are required, which need to be constantly replenished, increasing production costs and eventually rendering the process as economically unviable (Youssef et al. 2007). BSF are promising alternatives since some of these compounds present higher activity than synthetic surfactants, and only very low concentrations are needed for the process. BSF produced by bacteria generally present very low CMCs, especially when compared with

chemical surfactants (2100 mg/L, SDS), including surfactants used in EOR strategies, such as Enordet and Petrostep (both 1 g/L) (Pereira et al. 2013). However, production of BSF ex situ, and later transportation and deployment into the oil fields can still significantly increase oil production costs (Khire 2010). A more economical alternative is the production of BSF in situ which may eliminate the need of replenishment (Perfumo et al. 2010). MEOR strategies use much less energy than EOR, and are independent on the price of the crude oil (Sen 2008). One of the limitations of BSF-based MEOR is that added or locally-produced BSFs may be used as substrate by other bacteria (Chrzanowski et al. 2012) thus decreasing the efficiency of the process and the concentration of BSF may be a key factor in the outcome of the MEOR strategy. The search for new BSF producers is often connected to the development of new and improved MEOR techniques. However, as previously mentioned, most BSF producers have only been tested under aerobic conditions. As such, these producers may not be suitable for in situ application in the microaerobic and/or anaerobic environments present in most oil reservoirs (Perfumo et al. 2010). Anaerobic BSF producers can be seen as a viable solution of this problem (Perfumo et al. 2010; Zhao et al. 2015c). In addition to the advantages mentioned above, in situ strategies using BSF producing anaerobes are easier to implement than other MEOR strategies and do not require modification to the pipeline and equipment. Furthermore, depending on the lithography, bacteria can be spread by underground fluids and positively affect the oil recovery of a wider area (Zhao et al. 2015c). Since most oil reservoirs present high salinity levels (Simpson et al. 2011) and temperature (Nazina et al. 2007, 2008), anaerobes should also be halo- and thermotolerant. In some instances barotolerance may also be advantageous.

Most known BSF-producers under oxygen-limiting conditions have been isolated from oil wells and/or aimed at application in MEOR strategies (Javaheri et al. 1985; Yakimov et al. 1997; Castorena-Cortés et al. 2012; Gudiña et al. 2012; Zhao et al. 2015d). Many of these isolates use the petroleum hydrocarbons as carbon sources (Zheng et al. 2012; Gudiña et al. 2012; Wenjie et al. 2012). An advantage of using hydrocarbon-degrading BSF-producing bacteria in MEOR strategies is that no external carbon source is needed. In water flooding experiments in sand packed columns, strategies involving production of BSF by *R. ruber* Z25 ex situ were more effective than in situ strategies, with initial oil recoveries of 17–28% and 9%, respectively. This is thought to be due to low availability of terminal electron acceptor (Zheng et al. 2012). In 100-day crude oil degradation assays with *G. pallidus* H9, decreases in the aromatic, asphaltene and non-hydrocarbon fractions were observed in both aerobic and anaerobic conditions. Because the hydrocarbon fractions taken up by bacteria were not the same in both conditions, BSF composition was also affected, as mentioned previously. Furthermore, cell surface lipophilicity was affected by BSF production, which impacts interactions between cells and oil. In aerobic conditions, higher cell surface lipophilicity was observed when compared to anaerobic conditions, indicating an increased bioavailability of crude oil in aerobiosis. Crude oil viscosity and wax appearance temperature (temperature at which paraffin crystalizes) also decreased throughout the experiment (Wenjie et al. 2012). This was attributed to the preferred degradation of long-chained hydrocarbons by the bacteria under both aerobic and anaerobic conditions. While BSF can contribute to crude oil mobility, the metabolization of long-chain alkanes can help eliminate wax from the reservoir and production equipment, increasing oil fluidity and reducing operation costs and equipment damage. In anaerobic flooding experiments with *G. pallidus* H9 in sandstone packed columns, previously subjected to an initial EOR by water flooding, 7% of added oil was recovered after a second water flooding, corresponding to the MEOR (Wenjie et al. 2012). Although isolated from oil well injection water, *B. mojavensis* JF-2 did not use crude oil as substrate, utilizing instead water-soluble substrates and it was not inhibited by petroleum hydrocarbons. These characteristics, in addition to its ability to grow and produce BSF under ranges of temperature, pH and salinity, represent major advantages of this strain as a BSF producer for MEOR applications (Javaheri et al. 1985). In anaerobic core displacement experiments at high pressure (7 MPa), in situ growth of *B. mojavensis* JF-2 resulted in a recovery of 23% of residual oil which was attributed to BSF production (Marsh et al. 1995). In the same experiment, a mutant unable to produce BSF resulted only 6% oil recovery. *B. mojavensis* JF-2 has been patented (McInerney et al. 1985) and tested with some success in a field trial (McInerney et al. 1990). *P. aeruginosa* SG was

isolated and characterized with the aim of being used in in situ BSF production MEOR strategies. In an anaerobic core flooding test mimicking reservoir conditions, a flooding inoculated with *P. aeruginosa* SG increased oil recovery by 8.33% of initial oil, resulting in a total recovery of 63.94% of all initial oil (Zhao et al. 2015d). In a simulated MEOR anaerobic laboratory experiment conducted at 70 °C, a mixed culture composed mainly of *Thermoanaerobacter* sp. was able to remove 12% of the total heavy oil from carbonate porous media. BSFs were produced by the bacteria during the experiment (Castorena-Cortés et al. 2012). These results indicate that in situ MEOR strategies with BSF producers may be feasible in high-temperature (70 °C) and high pressure (14.2 MPa) reservoirs even in those with low available O_2 and with heavy oils, high viscosity and density.

One of the first in situ studies to present economic feasibility of a MEOR strategy involving production of BSF used the facultative anaerobes *Bacillus* RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049 (Youssef et al. 2007). Bacteria were inoculated into two limestone oil wells together with a supplement broth containing glucose. In other two oil wells only the supplement broth was added and a fifth well was used as negative control. Information on the concentration of oxygen was not provided, although there was evidence of anaerobic metabolism in inoculated wells. While BSF producers were found in the injected fluid of all five wells, BSF producers physiologically similar to the *Bacillus* were the only BSF producers recovered from the two inoculated wells. BSF production was observed only in the inoculated wells but the concentration of *Bacillus* cells remained close to the initial values. The results were interpreted as an indication that both *Bacillus* strains were metabolically active, due to the supplemented broth which favored their metabolism instead of that of indigenous bacteria. Overall the results were considered a success, with concentrations of BSF much higher than their CMC being produced in the inoculated wells. Inoculation with the BSF-producing strain caused an average increase of oil production of one barrel per day, and the effect persisted up to 7 weeks after inoculation. From an economic point of view it was estimated that the MEOR strategy in this study had a cost of $1.6 per barrel (Youssef et al. 2007). A subsequent study performed under the same conditions but with an increased volume of broth and inoculum added to the wells, confirmed the cost-effectiveness of the process (Youssef et al. 2013).

Biosurfactant production in high temperature reservoirs has also been reported (Nazina et al. 2007, 2008). In a MEOR strategy involving injection of air-supplemented water mixture in an oil well in China with stratal temperature of 60 °C, BSFs were detected together with an increase in aerobic and anaerobic bacteria. After 6 months of treatment, the concentration of methanogens and SRB returned to initial values.

A study using genetic markers to search for endogenous BSF producers in several American reservoirs found surfactin/lichenysin producing *Bacillus* in 8 out of 9 wells (Simpson et al. 2011). While genetic markers for the production of rhamnolipids by *Pseudomonas* were also searched for, no BSF producing Pseudomonads were detected. The closest relatives to the endogenous *Bacillus* were *B. licheniformis*, *B. mojavensis*, *B. sonorensis* and *B. subtilis* subsp. *subtilis* (Simpson et al. 2011). This together with other previously mentioned *Bacillus* isolated from these environments (Table 1) indicated that they are common BSF producers in oil reservoirs and Pseudomonads, on the other hand, seem to be rather scarce.

Hydrogen sulfide, a product of SRB metabolism, is often present in oil wells (Hasegawa et al. 2014). This compound is responsible for oil souring and infrastructures corrosion (Wolicka and Borkowski 2007). Since sulfide is known to inhibit BSF production under reservoirs conditions, its presence is of particular concern for MEOR strategies involving in situ BSF production (Zhao et al. 2015b, 2016a). A strategy involving a co-culture has been proposed, where one bacterial strain removes sulfide while another, *P. aeruginosa* SG or WJ-1, is responsible for anaerobic BSF production under low sulfide concentrations (Zhao et al. 2015b). An easier to control and simpler approach involved the genetically modified *P. stutzeri* Rhl which is simultaneously able to inhibit SRB growth, remove sulfide and produce BSFs under anaerobiosis and up to sulfide concentrations of 33.3 mg/L (Zhao et al. 2016a). These strategies do not only inhibit SRB development in oil reservoirs by having the exogenous bacteria successfully outcompeting them for nutrients, but also achieve the removal of some of the sulfide already present at oil reservoirs, thus increasing the quality of oil, decreasing associated carrion problems and specially, improving of the efficiency of MEOR strategies involving in situ BSF

production (Zhao et al. 2015b, 2016a). In general, these results highlight the applicability of anaerobic BSF production in MEOR, but also the importance using strains adapted to the reservoirs conditions for attaining increasingly efficient and cost-effective MEOR applications.

5.3 Production of gas hydrates

Gas hydrates are crystalline structures of similar appearance to ice and are constituted by trapped gas molecules surrounded by bonded hydrogens present in water. They are of particular interest for storing a great volume of natural gas per volume of gas hydrate and are found in the subsurface of the ocean-floors. BSFs, independently of their type, have been found to lower induction time and increase formation rates of gas hydrates in the ocean-floor (Rogers et al. 2003). Anionic BSF have been found to work as catalysts in the formation of gas hydrates by decreasing induction time, increasing formation rates, promote crystallization and not being consumed in the process. This results from the attraction of water molecules to the hydrophilic moiety of BSFs and the hydrocarbon gases to the hydrophobic part. Therefore, both compounds are brought together and induction time is reduced (Zhang et al. 2007). The formation of micelles may also aid the formation of gas hydrates (Rogers et al. 2003; Zhang et al. 2007). Small amounts (ppm range) of the BSF influenced gas hydrates formation (Rogers et al. 2003). BSF producers, namely *P. aeruginosa* and *B. subtilis*, have been found in anaerobic mud of the gas hydrate stabilizing zone (Lanoil et al. 2001). To test the effect of bacterial BSFs on the formation of gas hydrates in the ocean sediments, Zhang et al., (2007) tested anaerobic surfactin in sand packed columns. The surfactin produced by *B. subtilis* under anaerobic conditions was much more effective in catalyzing the induction and formation of gas hydrates than aerobic-produced commercial surfactin, with an increase of 216% of formation rate (Zhang et al. 2007). This was attributed to a difference in isoform mass proportions in both BSFs, with some isoforms, more predominant in the anaerobic BSF, presenting a stronger catalytic effect. The morphology of the gas hydrates was also shown to be affected by the BSF used. Large nodular hydrates developed in the presence of the anaerobic-surfactin, while small grainy hydrated developed in aerobic-surfactin experiments (Zhang et al. 2007).

5.4 Industrial BSF production and other industrial applications

During industrial production of BSF, oxygenation and agitation, used to enhance oxygen transfer rate, usually cause severe foaming. This results in problems of overflowing and contamination of tanks or equipment adjacent to the bioreactor, by rising and spreading of foam. Mechanical anti-foam strategies may not always work because of the amount of foam, and chemical strategies may inhibit bacterial growth. Microaerobic or anaerobic conditions represent a valuable industrial alternative to reduce or eliminate the foaming problem and only few cases of problematic foaming under anaerobic conditions have been reported (Ganidi et al. 2009; Boe et al. 2012; Pinzon et al. 2013; Kougias et al. 2014a, b).

BSFs are used in industry for their antiadhesive, antimicrobial and emulsifying properties. However, BSF are relatively expensive. A possible strategy to cut production costs is to use by-products as substrate for BSF production (Nitschke and Costa 2007). BSF can also be used in industry to promote the degradation of unwanted wastes and production of value-added products. In a study involving production of methane by anaerobic biodegradation of lipids from milk-fat, the synthetic surfactant sodium dodecylbenzenesulfonate (SDS) was added as an emulsifier (Petruy 1997). Since SDS can be toxic to the fermentative bacteria only small concentrations are tolerated (Petruy 1997). BSF are known to be more tolerated, thus their use instead of surfactants would likely increase production of compounds of interest. The addition of a BSF producer, instead of a synthetic surfactant, is also expected to lower production costs and reduce the need for continual addition of surfactant.

6 Conclusion

Aerobic BSF production is widely documented in scientific literature. However, the demonstration that bacteria able to produce BSFs in aerobic conditions may not be able to do it in anaerobic conditions and even if they are, the resulting BSF may be chemically and functionally different from that produced aerobically, has led to a growing interest in the understanding of the biological processes underlying anaerobic BSF production.

6.1 Biosurfactant producers

Most of the known BSF producers under microaerobic or anaerobic conditions are NRB, mainly belonging to the *Bacillus* and *Pseudomonas* genera. Anaerobic BSF production was reported to be represented in other genera of NRB, namely, *Bretibacillus, Rhodococcus* and *Geobacillus*. Regarding other metabolisms, so far, only eight bacteria using organic compounds as terminal electron acceptors, belonging to *Bacillus*, *Clostridium*, *Thermoanaerobacter* and *Anaerophaga* genera, and two SRB, *Desulfovibrio* and *Thermoanaerobacter*, have been identified as being able to produce BSFs under oxygen-limiting conditions. It is not clear if NRB are the predominant group of bacteria able to produce BSF under anaerobiosis and microaerobiosis or if this fact is the result of an increased focus in the search of BSF-producing NRB and comparatively few studies regarding SRB, fermentative bacteria or bacteria using other terminal electron acceptors. Despite many of the bacterial strains being isolated from oil, they have also been found in other environments, such as soil and sewage sludge. This indicates that bacteria able to produce BSF under oxygen limitation are ubiquitous even if present in low concentrations or with little diversity. However, to the best of our knowledge, many bacteria able to produce BSFs under aerobic conditions or isolated from anaerobic and microaerobic environments are never tested as possible microaerobic or anaerobic BSF producers. Therefore, it is reasonable to speculate that this represent a small fraction of the total diversity of bacterial populations able to produce BSF under microaerobic and anaerobic conditions.

6.2 Biosurfactants produced in oxygen-limiting conditions

BSFs produced in microaerobiosis or anaerobiosis have been identified as rhamnolipids, surfactins, lichenysins A and B, iturins, fengycin and glycolipids. Rhamnolipids are produced by *Pseudomonas,* an unspecified glycolipid was detected in *Rhodococcus* and all other identified BSFs are *Bacillus* metabolites. However most of the BSFs produced under oxygen-limiting conditions are still unidentified and uncharacterized, limiting the perspective for possible applications of these BSFs and the comparison between the performances of BSFs produced under aerobiosis and anaerobiosis. An enlargement of the pool of known anaerobically-produced BSF is expected in the near future.

6.3 Metabolic pathways involved in oxygen-limiting BSF production

Little is known about the metabolism of anaerobic or microaerobic BSF production, even though often BSF with different characteristics are produced in either anaerobic or anaerobic conditions. Further transcriptome and proteome studies should be performed to identify possible causes and differences between metabolisms in both conditions. Furthermore, genetic approaches such as mutagenesis may help identify relevant genes associated to BSF production in oxygen-limiting conditions.

6.4 Future perspectives

Considering the small number of isolates obtained so far and the scarcity of information on anaerobic or microaerobic bacterial BSF production, future research should focus on the prospection for new isolates and their characterization. Research on growth conditions and on the metabolic pathways involved in BSF production under oxygen-limiting conditions will also prove fundamental for maximizing the application of these bacteria in industrial and environmental contexts.

In general, comparing aerobic and anaerobic conditions, the latter are associated with longer lag times and slower growth. Additionally, biomass and BSF yields are also usually lower. Nonetheless, optimization of cultivation condition may reduce BSF production costs, making their applications economically viable. Likewise, understanding the interplay of factors affecting BSF production is also vital for the success of some of the more promising applications of anaerobic BSF producers, namely bioremediation or MEOR strategies.

Anaerobic or microaerobic BSF producing bacteria have been successfully applied in bioremediation and MEOR strategies and their potential for production of BSF and gas hydrates has been demonstrated. Because of the differences in the BSF produced by the same strain under different oxygen conditions it is also possible that further investigation of facultative anaerobes or anaerobes able to produce BSF may

result in an increase in the variety of available BSF, opening new perspectives for their applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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