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Compartive Studies and Production of Biosurfactant by *Bacillus tequilensis* KM15 from Marine/Salt Water /Brackish Water Species and Freshwater Fish Wastes as a Substrate

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Abstract: In this study, isolation, and screening of potential biosurfactant activity by *Bacillus tequilensis* KM15 from freshwater and marine water fish waste samples (scales) were successfully done. Amongst the 15 fish waste samples, 1 from freshwater (Mrigal carp) scales and 1 from marine water fish (Indo pacific king Mackerel) scales were selected for the optimization of enhanced biosurfactant production activity. The highest and most promising biosurfactant producers is freshwater fish (Mrigal carp) scales. Further studies like optimization of temperature, pH, Scales percentage, enhanced nutrient (Carbon and Nitrogen) supplements showed significant results by freshwater scales with *Bacillus tequilensis* KM15 treated samples. When compared to marine fish waste with freshwater fish waste samples, the freshwater fish waste (Mrigal carp) samples showed more biosurfactant productivity. The optimum values were pH at 7 (466 mg/l), Temperature at 30°C (482 mg/l), Scales percentage at 3% (450 mg/l), Nitrogen and Carbon at 15 g/l (482 and 426 mg/l) which showed shown enhanced biosurfactant production. Therefore, *Bacillus tequilensis* KM15 can potentially produce enhanced levels of biosurfactant under these optimized conditions by using fish waste samples as a substrate.

Keywords: Biosurfactant, Marine water, Freshwater, Fish waste, Scales, *Bacillus tequilensis* KM15

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Introduction

Microbial surface-active substances also referred to as biosurfactants, have attracted the attention of researchers recently. Despite the wide range of compositional structures in biosurfactants, these substances are always acknowledged to be amphiphilic molecules containing combined

hydrophilic and hydrophobic regions (Marchant *et al*., 2012). Because of this specific organization, these substances may concentrate at the interfaces of immiscible phases like air/water and oil/water, reducing surface and interfacial tensions. The physicochemical characteristics of biosurfactants, such as emulsifying capacity, mobilizing and dissolving agents, detergency, creating foam, wetness ability, and phase dispersion, are determined by their capabilities to lower surface and interfacial tension. These characteristics make them desirable molecules with potential uses in various industrial fields, such as the food and oil industries, domestic tasks, and the restoration of polluted regions (Mnif *et al*., 2015; Usman *et al*., 2016; Harikrishnan *et al*., 2021, 2022, 2023). They also make them available as more affordable and environmentally friendly techniques.

In comparison to their chemical counterparts, biosurfactants have several advantages, such as ecological acceptability, biocompatibility, digestibility, and efficiency under harsh pH, temperature, and salinity conditions, along with their functional specificity and the potential to be synthesized from renewable and less expensive substrates (Montero *et al*., 2015, Basha *et al*., 2022, 2023).

Since their discovery, microbial tensioactive substances have been the subject of extensive investigation on a global scale (Randhawa *et al*., 2014). However, most research (Andrade *et al*., 2015; Eraqi *et al*., 2016) have relied on the capacity of bacteria and yeasts to synthesise biosurfactants utilizing inexpensive substrates. Few studies have also noted the probable ability of filamentous fungi to produce biosurfactants. Using crude glycerol (CG) and corn steep liquor (CSL) as regenerative substrates to produce biosurfactants, the biotechnological advantages of the new strain *Rhizopus arrhizus* UC 1607 extracted from Caatinga soil (PE, Brazil) were examined in the current study (Silva *et al*., 2018; Pele *et al*., 2018).

When native inhabitants of places are polluted by petroleum or other chemicals containing hydrocarbons, biosurfactant-producing bacteria can be isolated from those areas. They can easily flourish in these contaminated surroundings while metabolizing and degrading the hydrocarbons they use as a carbon source (Santhini *et al*., 2014). Microbes can produce a wide range of chemicals to aid in metabolizing these intractable hydrocarbons. These molecules are formed as a component of the cell membrane of the microorganisms or are expelled extracellularly. They are amphiphilic surface-active substances. These compounds have a polar (hydrophilic) component and a non-polar (hydrophobic) group making up their structure. Mono-, oligo-, or polysaccharides, peptides, or proteins comprise the hydrophilic group, whereas saturated, unsaturated, and hydrogenated fatty acids or fatty alcohols typically comprise the hydrophobic moiety. According to (Pacwa *et al*., 2011), these substances can be broadly categorized into two classes: biosurfactants, which are low-molecular-weight chemicals, and bio emulsifiers, which are high-molecular-weight polymers.

In addition to enhancing the surface area and accessibility of hydrophobic, water-insoluble substrates, biosurfactants also function as quorum sensors, bind heavy metals, and promote biofilm development. Biosurfactants outperform synthetic surfactants in terms of surface activity, toxicity, biodegradability, and environmental compatibility (Wei *et al*., 2005). Although artificial surfactants and dispersants containing chemicals were frequently utilized to treat hydrocarbon pollution, long-term use of these substances frequently results in environmental devastation. Therefore, the presence of native microorganisms that can use hydrocarbons as a carbon source could potentially aid in the natural breakdown of hydrocarbon. This would increase the likelihood that bioremediation of hydrocarbon-contaminated sites utilizing microorganisms that produce biosurfactants will be implemented (Parthipan *et al*., 2017).

Biosurfactants, a category of surface-active,

amphiphilic chemicals with a wide range of structural variations generated by various microbes, have many uses in preventing oil pollution. They outperform chemical surfactants in particularity, low harmful effects, high biodegradable properties efficiency at high and low temperatures, pH and salinity, and broad applicability. They can be utilized to reduce oil pollution and help emulsify and degrade oily waste (Mukherjee *et al*., 2006). However, their limited commercial production is due to their high production costs, which are 3–10 times more than chemical surfactants. Recently, numerous inexpensive substrates have been examined by various researchers to generate biosurfactants (Henkel *et al*., 2012). Several of the raw materials that have been employed to lower production costs include animal fat, molasses starches, industrial garbage, olive oil mill effluent, agro-based goods including wheat bran and rice bran, and detergent stocks milk production waste (Makkar *et al*., 2011).

The formation of biosurfactants was examined in this study using a variety of deoiled cakes, fruit juice wastes, fresh fish wastes, and shrimp shell waste. It is also required to have a productive microbe for producing biosurfactants in order for them to compete with synthetic surfactants. Biosurfactants produced by marine bacteria are believed to be more efficient because the bulk of applications are in marine ecosystems (Muhammad *et al*., 2018).

Additionally, certain unique structural and functional traits are seen in marine microorganisms. Marine microbes create a number of high molecular weight polymers and glycolipid-type biosurfactants and emulsifiers that have significant potential use in numerous industries. For the generation of surface-active biosurfactant and bioemulsifier molecules, numerous marine bacteria have been investigated, including *Acinetobacter*, *Arthrobacter*, *Pseudo-monas*, *Myroides*m *Alcanivorax*, *Rhodococcus*, and *Halomonas*. Therefore, marine ecosystems offer a great chance to choose powerful bacteria (Ovissipour *et al*., 2008).

In this study, *Bacillus tequilensis* KM15-treated freshwater fish scales and marine fish scales were isolated and screened for potential biosurfactant-producing activity. The best samples with optimal conditions for producing higher levels of biosurfactant were further researched for optimization and compared.

Materials and Methods

Production and extraction of biosurfactant using synthetic media:

Ten bacterial isolates were injected and incubated individually at 37 °C for 48 h in 250 ml of each media. Two replications were used in the experiment. After 48 h, cell-free supernatant was obtained by centrifugation at 10,000 rpm; supernatant was then precipitated by bringing pH to 2.0 with 6 N HCl; and stored overnight at 4°C (Satpute *et al*., 2010). The precipitates were separated by centrifugation at 12,000 rpm for 20 min, followed by two times methanol extractions.

Biosurfactant production and extraction:

In a 250 ml Erlenmeyer flask, bacterial isolate KM15 was cultured for 48 h at 200 rpm in 100 ml MSM before being centrifuged for 15 min at 10,000 rpm. By lowering the pH to 2.0 with 6 N HCl, the supernatant was precipitated and left to stand overnight at 4°C. Centrifugation at 12,000 rpm for 20 min yielded the precipitate, which was then twice extracted with methanol (Maneerat *et al*., 2005).

Optimization studies:

Carbon substitution medium:

Each type of fish waste was assessed as a potential carbon source for making biosurfactants. In the traditional medium, sucrose was employed as an improved carbon source at various concentrations ranging from 5 to 25g/l. The added mineral salts and trace elements have the same chemical make-up as the standard biosurfactant manufacturing medium. By assessing the biosurfactant

production capability, the ideal carbon source to improve the biosurfactant is identified.

Nitrogen substitution medium:

Each type of fish waste was assessed as a potential supply of nitrogen for the synthesis of biosurfactants. The concentration of NH_4SO_4 employed as the nitrogen source in the traditional medium ranged from 5 to 25 g/l. The added mineral salts and trace elements have an identical chemical make-up as the standard biosurfactant manufacturing medium. The ideal nitrogen source to improve the biosurfactant is identified by assessing the biosurfactant production capability.

Environmental conditions (*Temperature and pH*):

Two created products' optimized biosurfactant production was assessed as variations in surface activity in response to various environmental variables (temperature and pH). For every biosurfactant item the pH effect was determined by employing 1 N NaOH or 1 N HCl to change the stock solution's pH value (5, 6, 7, 8 and 9). Each stock solution was incubated for 120 min at various temperatures ranging from 20, 25, 30, 35, and 40℃ to assess the biosurfactant's heat resilience.

Results and Discussion

The production of biosurfactants from marine and freshwater fish waste samples (scales) taken from two separate study regions was improved through enrichment culturing. In total, 15 freshwater and 5 marine water fish waste samples (scales) were collected and used as substrate for the biosurfactant production by inoculating the *Bacillus tequilensis* KM15. All the samples were produced biosurfactants. Maximum biosurfactant produced samples were selected for further optimization studies.

Two samples, *Cirrhinus mrigala* (Mrigal carp) from freshwater fish waste sample and *Comberomorus guttatus* (Surmai or Indo pacific king Mackerel) from marine water fish waste samples were chosen for further studies based on the results of biosurfactant production capability (Tables 1, 2).

From the above samples, the best biosurfactant producing fish waste sample from fresh water is *Cirrhinus mrigala* commonly known as Mrigal carp with 326± 1.04 mg/l. Whereas in marine water fish waste sample is *Comberomorus guttatus* commonly known as Surmai (Indo pacific king Mackerel) with 228± 1.06 mg/l, respectively.

The above two fish waste samples have been chosen for further studies, like optimization of biosurfactant production capacity by varying the environmental conditions like temperature and pH.

Effect of Temperature on Biosurfactant production:

To determine the optimum temperature for enhanced biosurfactant production the bacterial culture *Bacillus tequilensis* KM15 treated freshwater fish scales and marine water fish scales were used as a substrate and tested with various temperatures from 20 to 40°C (Fig. 1). The optimum temperature was observed as 30°C, which produced 482 mg/l from the freshwater fish scales and 385 mg/l from the marine water fish scales. While comparing both it was observed that the freshwater scales showed enhanced biosurfactant production.

As per the Wei *et al*. (2007), the isolate was shown to be sensitive to temperature changes, and its growth and generation of biosurfactant were both limited to temperatures between 25 and 45°C. At 30°C, it produced its best results. Most of the research also state that the ideal temperature falls between 30-37°C.

Effect of pH on Biosurfactant production:

The bacterial culture *Bacillus tequilensis* KM15 treated freshwater fish scales and marine water fish scales were tested with different pH levels from acidic to alkaline. The different levels of the pH form 5, 6, 7, 8 and 9 were tested to know the optimum pH levels to enhance the biosurfactant. The results revealed that the pH level 7 is the most optimum temperature for freshwater fish scales

Table 1: Screening of freshwater fish wastes (scales) as substrates for BS production by *Bacillus tequilensis* KM15

Fish variety (Common name and Abbreviation used)	Scientific name	Biosurfactant production (mg L^{-1})
Grass carp (GC)	*Ctenopharyngodon idella*	169 ±1.07
Kalbasu (KB)	*Labeo calbasu*	250 ± 1.09
Roopchand (RC)	*Piaractus brachypomus*	317 ± 1.07
Mrigal carp (MC)	*Cirrhinus mrigala*	326 ± 1.04
Bocha (B)	*Labeo catla*	247 ± 0.99
Murrel (M)	*Channa straitus*	237 ± 1.1
Tilapia/Cichlid fish (T)	*Oreochromis niloticus*	121 ± 0.96
Common carp (CC)	*Cyprinus carpio*	145 ± 0.95
Reba (R)	*Cirrhinus reba*	119 ± 0.98
Catla/Indian carp (IC)	*Catla catla*	137 ± 1.02
Rohu/Ravata (RR)	*Labeo rohita*	222 ± 1.06
Tor Tor (TT)	*Mahseer*	197 ± 1.01
Hilsa (H)	*Ilish shad*	104 ± 1.01
Pedda parka (PP)	*Osteochilichthys brevidorsalis*	134 ± 0.92
Pool barb (PB)	*Puntius sophore*	106 ± 1.03

Table 2: Screening of Marine/salt water brackish water species Fish wastes as substrates for biosurfactant production by *Bacillus tequilensis* KM15

S. No.	Fish variety (Common name)	Scientific name	Biosurfactant production (mg L^{-1})
1	Barramundi – (Asian Sea bass)	*Lates calcarifer*	214± 1.02
2	Surmai (Indo pacific king Mackerel)	*Comberomorus guttatus*	228± 1.06
3	Indian Salmon – (Rawas)	*Eleutheronema tetradactylum*	185±1.02
4	Fingerscale sardine	*Sardinella fimbriata*	112± 1.02
5	Indian oil sardine	*Sardinella longiceps*	139±1.09

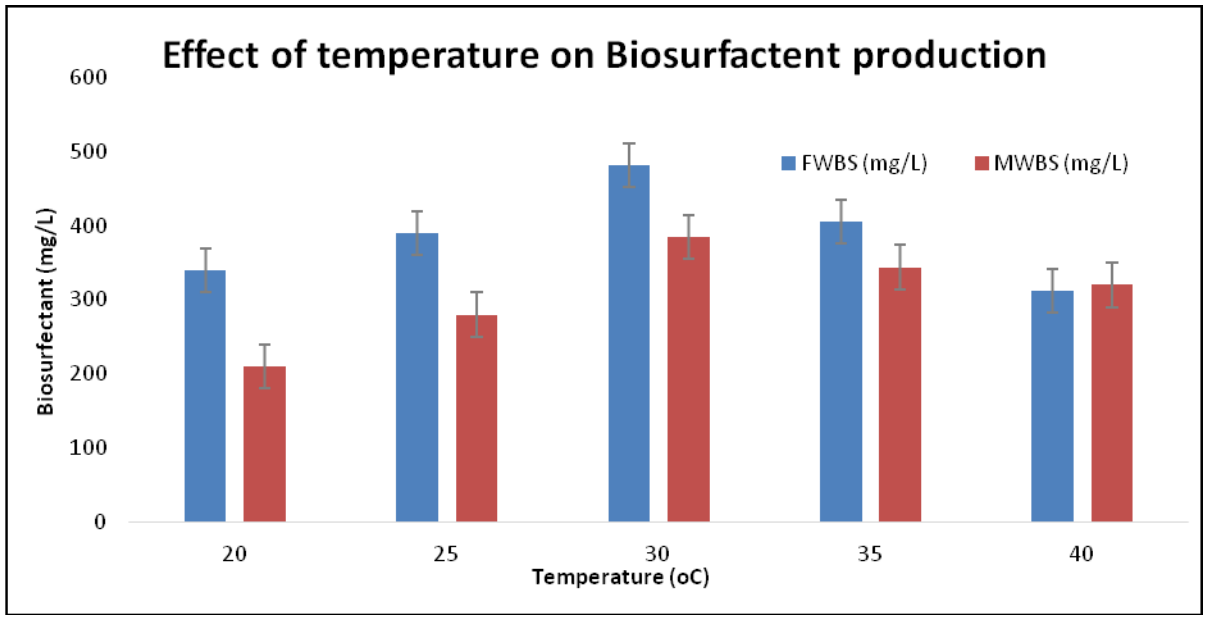

Fig. 1: Effect of Temperature on Biosurfactant production.

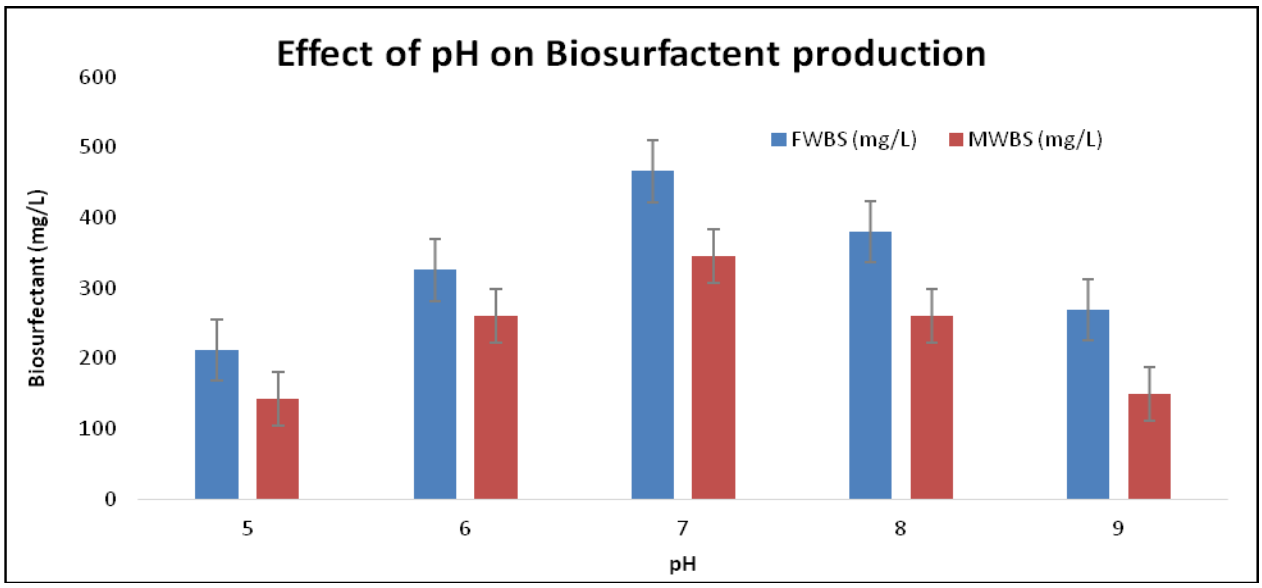

Fig. 2: Effect of pH on Biosurfactant production.

with 466 mg/l and marine water fish scales with 345 mg/l biosurfactant production activity (Fig. 2). By comparing both the freshwater fish scales show enhanced levels of biosurfactant production activity.

At a pH of 7, pH and its best action are found. Numerous studies have shown that *Pseudomonas* species thrive best when the pH is between 6 and 7. Additionally, it has been suggested that the pH of the surrounding environment may impact the shape and size of the tiny particles in a water-oil system (Das *et al*., 2009). According to (Gudiña *et al*., 2010), groups with negative charges at the ends of biosurfactant molecules cause instability in acidic environments.

According to Zhu *et al*. (2020) study, the production of residues at deficient pH levels (i.e. pH = 2) reduced the capacity of the waste from fish-produced biosurfactant compounds to decrease surface tension. However, at pH levels between 4 and 12, the surface tensions of biosurfactants produced from fish waste remained essentially constant. These findings demonstrate the potential of the *Bacillus subtilis* N3-1P crude biosurfactant in a frigid maritime environment.

Effect of Scales percentage on Biosurfactant production:

The scales of the freshwater fishes and marine water fish were used as a substrate with culture media inoculated with *Bacillus tequilensis* KM15. The scales were used in different percentages to know the optimum percentage of the scales (Fig. 3). Both fish scales were taken from 1, 2, 3, 4 and 5% and tested for biosurfactant production activity. The results revealed that the 3% freshwater fish scales showed enhanced biosurfactant production (450 mg/l) when compared to marine water fish scales (320 mg/l).

For instance, increased valine and lysine concentrations could significantly improve the generation of biosurfactants, but alanine and arginine could decrease the ability of *Bacillus* strains to produce biosurfactants (Makkar *et al*., 2011). The extra nitrogen in the medium may cause the presence of biosurfactant synthesis at a greater peptone concentration.

According to Reis *et al*. (2013), inadequate nitrogen promotes the formation of biosurfactants. Constant cell development and division are impeded in nitrogen-limiting conditions. The expression of the gene that produces biosurfactants is then increased, and a microbial metabolism that favours the generation of additional metabolites is fostered (Nurfarahin *et al*., 2018).

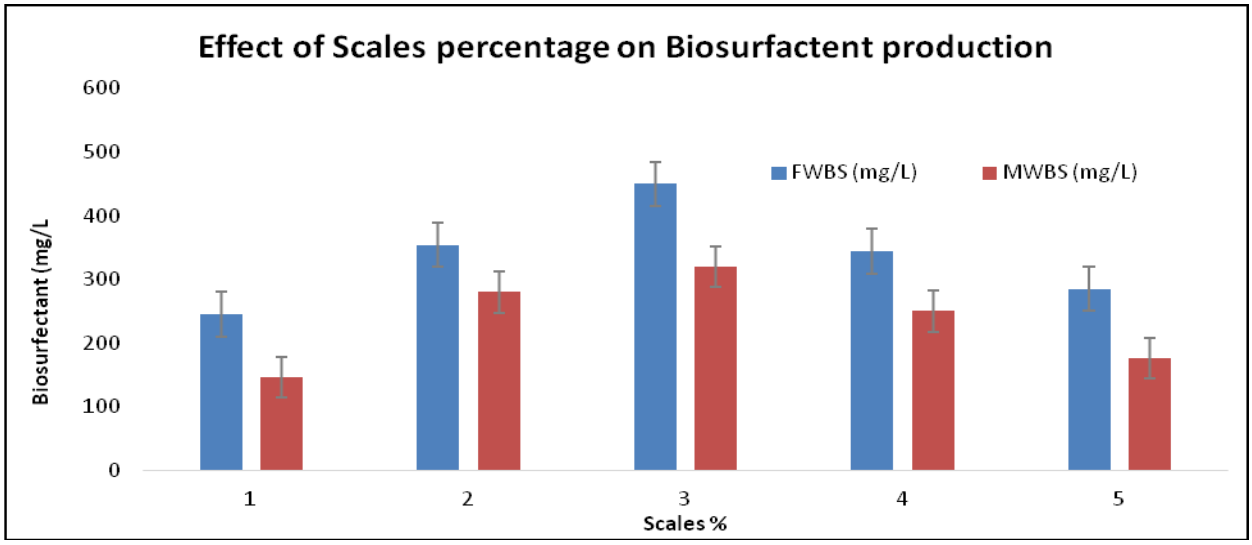

Fig. 3: Effect of Scales percentage on Biosurfactant production.

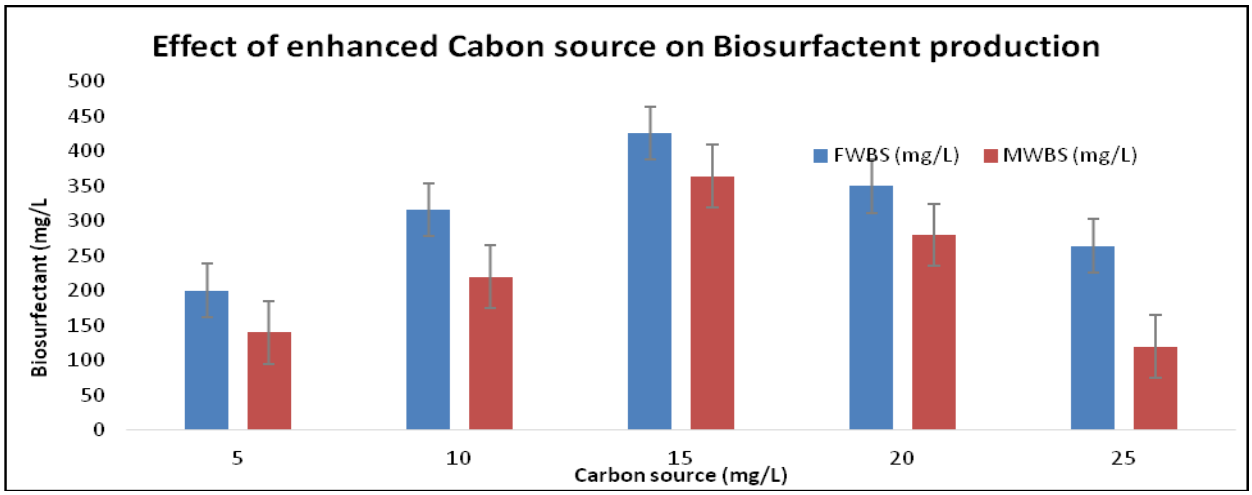

Fig. 4: Effect of enhanced Carbon source on Biosurfactant production.

Effect of enhanced Carbon source on Biosurfactant production:

The two samples were supplied enhanced quantities of sucrose as a carbon source as a substitute media to stimulate the biosurfactant production. The results revealed that the freshwater fish scales showed significant results when compared to marine water fish scales (Fig. 4). Best results were obtained at 15 g/l carbon source, for fresh water it was 426mg/L and for marine water fish scales it was 364 mg/l.

Pepi *et al*. (2013) found that using animal fat substrate as the only carbon source reduced the formation of biosurfactants due to the high percentage of palmitic acid (26.40%) and oleic acid (24.16%). Given that those fatty acids have also been commonly detected in waste from fish (Khoddami *et al*., 2009), this may assist in explaining why *Bacillus subtilis* N3-4P and N2-6P suppressed the formation of biosurfactants in the current investigation.

The carbon and nitrogen sources are two critical components for the creation of biosurfactants. Results were most significant with sucrose. The ideal sucrose concentration was 4% w/v. These findings confirm prior research by Persson *et al*. (1987), which demonstrated biosurfactant generation by *Pseudomonas* in the

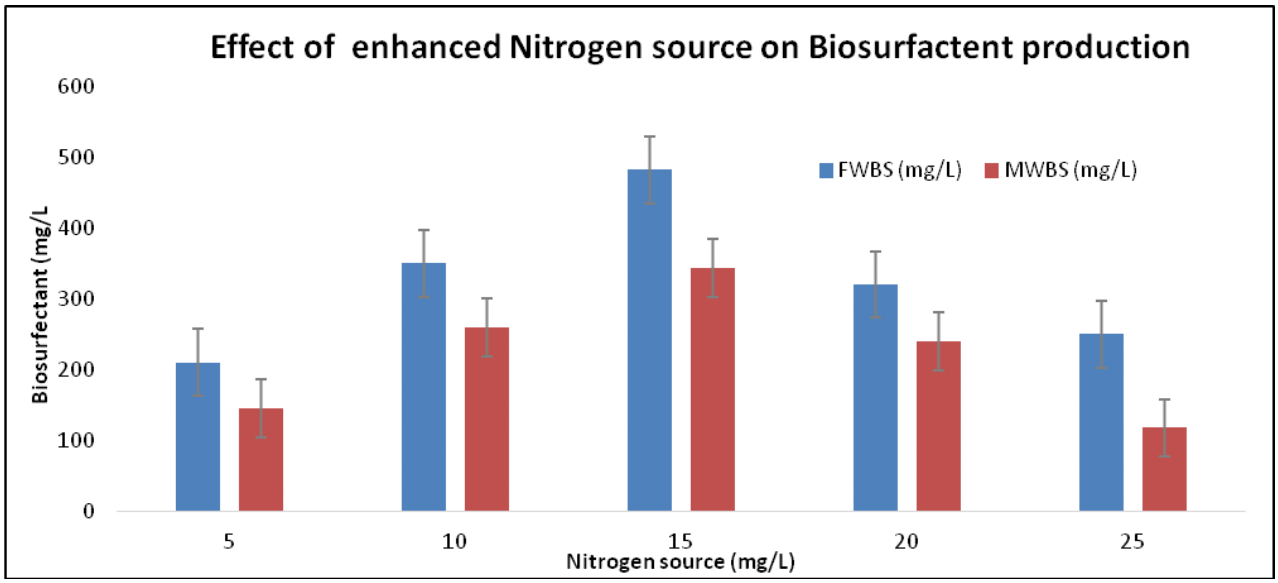

Fig. 5: Effect of enhanced Nitrogen source on Biosurfactant production.

presence of sucrose as well as favourable proliferation and production of biosurfactant when sucrose was used as a source of carbon (Persson *et al*., 1987; Bayoumi *et al*., 2011). However, when fish peptones were used as the carbon source, those strains responded in a different way. ST reduction, a biosurfactant production indicator, was only found in *Bacillus subtilis* N3-1P and *Bacillus subtilis* samples while employing FL(C) as the carbon source. Similar to this, for *Bacillus subtilis* N3-1P and *Bacillus subtilis*, the ST of FH(C) based culture medium decreased to 29.6 and 31.2 mN/m, respectively (Siegmund *et al*., 1991; Khopade *et al*., 2012). When employing fish peptones as the carbon replacement, *Bacillus subtilis* N3-1P produced the most biosurfactants. For development and metabolic processes, bacterial strains require organic carbon sources.

Effect of enhanced Nitrogen source on Biosurfactant production:

Similar to the above carbon source, the nitrogen is also supplemented NH_4SO_4 as a Nitrogen source and observed significant results with freshwater fish scales (482 mg/l), when compared to marine water fish scales (344 mg/l) (Fig. 5).

It is well known that sources of inorganic nitrogen produce better outcomes when used to produce biosurfactants. (Nansingh *et al*., 1999) Similarly achieved comparable results for *Pseudomonas* using ammonium sulphate. According to Hamzah *et al*. (2013), all organic nitrogen sources encouraged the formation of biosurfactants and maximized peptone emulsification.

Conclusion

The utilization of fish waste as a cheap substrate for *Bacillus tequilensis* KM15 growth to produce biosurfactants was investigated in this study. The samples from freshwater fish waste had a higher biosurfactant output than those from marine fish waste using the standard medium.

Moreover, this study optimized various parameters like pH, Temperature, Scales percentage, Carbon and Nitrogen nutrients substitution to enhance and compare the biosurfactant production capacity. Results revealed that the use of fish waste samples under optimized conditions can be able to produce enhanced levels of biosurfactants. This study demonstrated an effective approach for generating environmentally friendly biosurfactants production to reduce the environmental pollution.

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