Stenotrophomonas Maltophilia: Novel Biopolymer Producing Plant Pathogen

rashida rahmat zohra (rrxohra@gmail.com)
Jinnah university for women
AYESHA SIDDIQUI
Jinnah University for Women
BUSHRA SYED
Jinnah University for Women
MAHNOOR SALEEM
Jinnah University for Women
HEEBA SHAHID
Jinnah University for Women

Research Article

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Abstract

Biopolymers are making their way into the global market very rapidly owing to their biodegradability and environmentally friendly characteristics. Xanthan, a microbial polysaccharide is being produced by plant pathogens. It is widely used in numerous industrial applications. In the current study, plant pathogenic bacterial strains were isolated from infected plants and screened for biopolymer production. Several strains having higher yield of polymer were further subjected to the physicochemical and genomic analysis. The highest biopolymer producing strain *Stenotrophomonas maltophilia X2* was found to be novel in commercial exploitation for biopolymer production. It was concluded that, the strain *Stenotrophomonas maltophilia X2* can produce higher yield of biopolymer under static condition; in self formulated medium containing 5% sucrose at pH 7.0 when incubated at 28°C for 120 hours.

Introduction

Increasing cost, depleting petro fuel and environmental concerns involved in the production of synthetic and natural polymers have kicked the search for alternative sources for polymer production. For this purpose, various bio-based materials have been explored for the production of biopolymers (Mohsin et al., 2018). The biopolymer can be extracted from microbial and plant sources or can be synthesized chemically from various biological materials (Katherine et al., 2017). Significant considerations has been given to the biopolymers produced by microbes as it is easy to scale up, environment friendly and yield high quality product (Katherine et al., 2017).

A variety of microbial polymers such as xanthan, dextran, levan, alginate, pullulan, cellulose have tremendous applications in industry (Ates, 2015). These biopolymers display diverse range of physic-chemical properties and add value to commercial products. Many of the biopolymers are polysaccharide in nature and are produced as extracellular metabolite which are purified and used in numerous pharmaceutical, medical, food and research applications. Among variety of different polysachharides, Xanthan is an exopolysaccharide of glucose, mannose, and glucuronic acid monomers, produced by Xanthomonas species. Mostly the molecular weight of the polymer ranges from 2×10^6 to 20×10^6 Da, however low molecular weight molecules have also been reported (Sajna et al., 2015).

The unique physicochemical characteristics of xanthan enables it to be largely used as stabilizers, thickener, emulsifier and in various industries like pharmaceutical, food and petroleum because of its additional friction reducing capabilities (Carignatto et al., 2011). The resistance of xanthan to broad range of temperatures, pH and salts also makes it suitable for variety of industrial applications. (Nasrsoudi *et al*., 2007).

Xanthan based products are in a surge and an estimate is that 10–15% innovative xanthan based products have been introduced into the market annually and the demand is increasing tremendously. (Khan et al., 2016).

Xanthomonas species are plant pathogens and causes leaf blight. The isolated bacterium produces copious amount of polymer when suitable conditions and nutritional requirements are met (Rosalam et al., 2008), (Borges et al., 2008), (Rosalam *&* Vendruscolo, 2007). Different techniques are employed for the recovery of polymer including centrifugation, filtration, organic solvents and salts (Candia & Deckwer, 2008, Palaniraj & Jayaraman, 2008).

Current study was designed to explore the native xanthan producing microorganisms and their characterization for the production of polymer. The polymer obtained will be characterized and utilized in cosmaceutical formulations in future.

Material And Methods

Culture media formulation

A new culture medium was self-designed by modifying the media used by Rosalam et al., 2008 and Carignatto et al., 2011. Both the medias were studied and their effect were observed on standard strains of *Xathnomonas campestris* pv vesicatoria FCBP-PB-0003, *Xathnomonas campestris* pv mangiferae indica FCBP-PB-0003, obtained from First Fungal Culture Bank of Pakistan, Institute of Agriculture Sciences, University of Punjab, Pakistan and Xanthomonas *campestris* ICMP 8425, *Xanthomonas campestris* pv. campestris ICMP 21080 from Landcare Research Center NewZealand. The modified media, was named as Production Media (PM media).

Table 1
Composition of Nutrient Medium PM for xanthan production

S. No	Component	Production Media (gm/l)
01	Sucrose	50
02	Agar agar	20
03	Yeast Extract	20
04	K2HPO4	10
05	MgSO4	0.4
06	CaCO3	05
07	Bacto Peptone	30

Sample collection and isolation

Samples were collected from the diseased part of different plants leaves that were blighted. The infected plants of *brassica* family were sought from various parts of University of Karachi and local vegetable vendors. The samples were cut into small pieces and dipped in 70% ethanol to surface sterilize the leaves samples and then washed with sterile distilled water, air dried and placed the leaves pieces on PM media agar plate at 30°C for 24hrs (Borges et al., 2008). For culture purification, the bacterial cultures were re-streaked on fresh nutrient agar plate with 0.85% saline dilutions (Rosalam *&* Vendruscolo, 2007). Among all the isolates, higher polymer producing strain X2 was subjected to optimization of physicochemical parameters and selected for further study.

Biochemical characterization of strains

Isolated biopolymer producing strains were identification based on morphological and biochemical analysis according to "Berge's Manual of Determinative Bacteriology" and "Microbiology a Laboratory Manual by Cappuccino James G Sherman" (Bergey & Holt, 1993, Cappuccino and Sherman, 2002). The biochemical tests performed on the cultures included Gram's reaction, KOH string test, Starch hydrolysis, Tween 80 hydrolysis test, Citrate utilization, SIM (Sulfide, indole, motility) test, Catalase test and Urease test to differentiate between the *Xanthomonadacea sp.* and other biopolymer producing cultures.

Ribotyping and bioinformatics analysis: Phylogeny of the highest producing strains was confirmed by 16S rDNA analysis using the protocol as described earlier R.R.Zohra (Zohra *et al*., 2008), and the obtained nucleotides sequence was submitted to GenBank, NCBI with the accession ID: MK422148. Furthermore, the phylogenetic tree of *strain X2* was generated using software "MEGA-X (VERSION: X)" after the 16S rDNA analysis. Also, the timeline of the same bacterial strain was generated using the same software (Kumar, et al., 2018).

Operational conditions for biopolymer production

To get the maximum production of biopolymer, the inoculum was prepared by incubating the microbial strain for 24 hrs in PM media which is 5% of the volume of total fermentation media. Culture broth was then transferred into main fermentation flasks for further incubation and analysis of various fermentation parameters such as agitation, temperature, incubation period, pH and carbon concentration. All experiments were performed in triplicates (Candia & Deckwer, 2008). Each factor was studied separately, one at a time and after the completion of incubation, culture broth was subjected to downstream processing for the recovery of biopolymer.

Effect of incubation time

To check the effect of incubation time on biopolymer production microbial strain was incubated in optimum growth medium and conditions from 24–192 hrs.

Effect of Sucrose concentration

Isolated culture was inoculated in the production media by varying the sucrose concentration from 35-90g/L. The inoculum was transferred to the main culture flask and incubated for 120hrs at optimized fermentation conditions.

Effect of Temperature

To optimize the temperature for maximum biopolymer production the culture was kept in the range of 25–40°C for 120hrs as recommended by Carignatto et al., 2011.

Effect of pH

To optimize the pH, the culture was checked in PM media in the pH range of 5–9. To adjust the pH, 1N NaOH and 1M HCL were used before autoclaving the nutrient medium.

Effect of Agitation

To check the effect of agitation, inoculated flasks was incubated at shaking and non-shaking conditions at 30°C for 120 hours. The shaking was provided by keeping the culture flasks in orbital shaker at 80 rpm.

Downstream processing of biopolymer

Fermented culture medium was centrifuged after incubation at 10,000 rpm for 4 minutes at 4°C to separate the biomass from broth. The supernatant recovered was saved for the recovery of biopolymer. The biomass settled in the pellet was dried at 55°C for 24hrs to remove the moisture (Freitas, et al., 2009).

To recover the biopolymer from cell free fluid, chilled ethanol was added in 1:3 ratio in the supernatant over constant stirring. The content was left at -18°C (overnight) for complete recovery of the dissolved polymer from aqueous phase. Precipitates of biopolymer were separated from broth by centrifugation at 10,000 rpm for 4 minutes. The supernatant was decanted and pellet obtained of the polymer was dried in hot oven at 55–60°C for 24–48 hours. The amount of polymer recovered was estimated by the dry weight of the polymer per liter of the broth medium (Freitas, et al., 2009).

Results And Discussion

Screening and characterization of polymer producing strains:

In the present study 20 strains were isolated from the leaves of the plants that were blighted and produced yellow pigment demonstrating the occurrence of the species of the family *Xanthomonadaceae*. All the isolated plant pathogens were able to produce viscous polymer on nutrient agar and production media. However, only the yellow pigment producers were selected and recultured as *Xanthomonadaceae* family produce yellow pigmentation on nutrient agar and

growth media (Borges et al., 2008, Candia & Deckwer, 2008, Freitas, et al., 2009, Kumar, et al., 2018). Among the screened cultures, strain X2 showed the highest production and was selected for biopolymer production optimization studies (Fig. 1A).

Biochemical characterization:

Through phenotypic profiling and biochemical characterization, 8 strains were found to be gram negative and showed quite similar characteristics and were studied further for biochemical test analysis. However, some of the strains were slightly oxidase positive and H_2S negative unlikely as compared with the past literature of strains belonging to *Xanthomonadaceas* (Naqvi *et al.* 2012 and Arshad *et al.* 2013).

Table 2
Biochemical characterization of biopolymer producing plant pathogens

Strain	*Strenotrophomonas maltophilia strain X2*	*Bacillus cereus strain X4*	*Strenotrophomonas maltophilia strain X7*	*Alcaligenes faecalis strain X10*	*Strenotrophomonas sp. strain X12*	*Bacillus cereus strain X14*	*Bacillus sp. strain X15*	*Strenotrophor maltophilia st X22*
Genbank Accession	MK422148.1	MK775363	MK775463	MK775526	MK775529	MK780189	MK780182	MK782051
Gram's reaction	–	–	–	–	–	–	–	–
Citrate utilization	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Lactose	–	–	+	+	+	+	+	+
Maltose	+	–	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	-	+
Tween 80 hydrolysis	+	+	–	–	+	-	+	–
Urea utilization	+	+	–	–	–	+	+	–
Nitrate reduction	–	+	+	–	+	+	+	–
H2S production	–	–	–	–	–	-	-	–
Indole	–	–	–	–	–	+	+	–
Motility	+	+	+	+	+	-	-	–
MR	–	–	–	–	–	+	+	+
VP	–	–	–	+	–	-	-	–
KOH	+	+	+	+	+	+	+	+

Bioinformatics analysis:

All the strains were subjected to ribotyping and found to be belonging to different genera of bacterium (Table 2). Owing to high biopolymer production, Bacterial strain X2 was selected for further study on biopolymer production parameters rest of all strains were glycerol preserved for future studies. The strain X2 was characterized as *Stenotrophomonas maltophilia* belonging to family *Xanthomonadaceae*. 16S rDNA nucleotide sequence was compared with other related species sequences by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). It showed that the sequence is novel and similar to *Stenotrophomonas maltophilia* after multiple alignment. Nucleotide sequences of all strains were deposited in GenBank database and received accession number as listed in Table 2. This sequence of *Stenotrophomonas maltophilia X2* (MK422148) was further analyzed by phylogenetic tree construction (Fig. 3).

The *S. maltophilia strain X2* is genetically related to the *Stenotrophomonas sp. strain X22*. Basically, the *Stenotrophomonas sp. strain X22* is genetically subdivided into two phylogenic nodes (categories), one of which gives rise to the strain x12. And the other node gives rise to the clad that bears *S. maltophilia strain X7* and *S. maltophilia strain X*9, which are genetically close to *Stenotrophomonas sp. strain X12*.

The other node (15) of *Stenotrophomonas sp. strain X22* gives rise to the strain *S. maltophilia strain X2*, that are further subdivided to the strain *A. faecalis strain x10, B. cereus strain X4*, *Bacillus sp. strain X15* and *B. thuringienesis strain X14*, on the basis of small genetic differences. In these sub divisions, *Bacillus sp. strain X15* and *B. thuringienesis strain X14* are likely similar to each other. Whereas, *B. thuringienesis strain X14* and *Bacillus sp. strain X15* are likely similar to each other. The ancestor of strain *A. faecalis strain x10* is likely similar to strain *S. maltophilia strain X2*. On the other hand, *B. cereus strain X4* is similar to the ancestor of *Bacillus sp. strain X15* and *B. thuringienesis strain X14*.

Members of this species are known to display high genetic, ecological and phenotypic diversity, forming the so-called *S. maltophilia* complex (Smc). Heterogeneous resistance and virulence phenotypes have been reported for environmental Smc isolates of diverse ecological origin. According to the past literature regarding the phylogenic analysis of *stenotrophomonas maltophilia*, its genome is reported to have certain genetic matches with some species of bacillus and *Geobacillus stearothermophilus strain KCB 2.*

The evolutionary timeline of the *Stenotrophomonas maltophilia X2* can be seen in Fig. 4 which reveals its presence in several ages and its biological features, including the fact that, this strain was first found 1733 MYA (Million Years Ago). The presence of this bacteria covered several geological periods comprising on, meso-proterozoic, neo-proterozoic, paleozoic and mesozoic periods. These geological timescales showed a great variation in the levels of oxygen, carbon dioxide and solar luminosity, that were acceptable for the growth of *Stenotrophomonas maltophilia*

Optimization of Fermentation parameters

Stenotrophomonas maltophilia X2 was studied for production and biopolymer recovery parameters. Physicochemical parameters such as incubation time, temperature, pH, shaking/static and substrate concentration were analyzed one at a time. Each parameter was performed in a triplicate set.

Effect of incubation time

Incubation time is an important variable for biopolymer production. By varying the incubation period from 24 hrs to 192 hrs, it was found that at 120 hrs X2 yield highest biopolymer production and then gradual decrease was observed (Fig. 5). In the initial hours, the nutrients are expected to be maximally utilized for biomass increment; the polymer production being the secondary metabolite increased in later phases on incubation. After 120 hrs, the polymer production was found to be decreased which could be attributed to utilization of polymer as sugar reserve by the bacterium (Candia & Deckwer, 2008).

Effect of sucrose concentration

Sucrose was used as carbon source in the media as reported in literature (Zohra *et al*., 2008). To investigate the appropriate amount of sucrose required to produce highest yield of xanthan, different concentrations were checked i.e. 3%, 5%, 7% and 9% respectively. The best biopolymer production by X2 was found at 5% and it was observed that the growth and biopolymer production started to restrict after 5% (Fig. 6). In the past literature sugar concentration higher than 6% had negative influence on xanthan production and viscosity and 2–4% of carbon source is preferred as high concentration of sucrose inhibits the bacterial growth (Caroline & Claire, 2007, Swamy et al., 2012, Arshad et al., 2015)

Effect of pH

Fermentation process for biopolymer is slightly acidic because of the metabolism of nitrogen and sugar sources which produce various acids as end product (Zohra *et al*., 2008). The growth of cells is dependent on pH, hence affecting the biopolymer production (Caroline & Claire, 2007). The pH 7 was found to be sufficient for biomass and biopolymer production (Fig. 7). However, the pH below and above 7 were insufficient for the biomass and biopolymer production rate (Letisse et al., 2001, Bajaj et al., 2007, Kerdsup et al., 2009, Gumus et al., 2010, Soudi et al., 2011, Ozdal & Kurbanoglu, 2018).

Effect of agitation of fermentation media on biopolymer production

It was concluded that, the bacterial cells were affected by mechanical agitation negatively and were best grown at static condition. The static incubation conditions for biomass and biopolymer production were found to be sufficient (Fig. 8). However, according to past literature biomass and biopolymer production increases within agitation fermentation condition (Freitas, et al., 2009). While in this study the agitation fermentation condition showed inverse relationship with the xanthan production using strain X2 (Caroline & Claire, 2007, Soudi et al., 2011, Swamy et al., 2012).

Effect of temperature

The effect of temperature on biopolymer yield and growth of the organism were studied by optimizing the temperature between the ranges 25°C to 35°C.Ideal temperature for biopolymer production from the results obtained was 30°C at which both the biomass and polymer yields were highest (Fig. 9). These results were in agreement with the past literature (Candia & Deckwer, 2008, Gumus et al., 2010, Shu & Yang, 2010).

Downstream processing of biopolymer:

Biopolymer produced by the strain in response to supplied nutrients and fermentation conditions can be recovered from various techniques like ultrafiltration, precipitating agent, and enzyme treatment. Because of the high viscosity of fermented medium it is difficult to separate xanthan gum from unwanted constituents. High viscosity of fermented medium leads to degradation of polysaccharide during centrifugation (Suresh & Prasad., 2005). To be use as food additives it is necessary for xanthan gum to be free of biomass and recovery agents. Including separation of unwanted debris, it is also important not to effect the properties of xanthan gum, which can be effected adversely by heat treatment processes (Garcia, et al., 20000). It is a standard procedure for xanthan gum recovery to precipitate out polysaccharide using precipitating agent such as isoprapanol, isobutanol (Nasr, et al., 2007). Other oragnic solvents can also be used like methanol, ethanol, t-butanol and acetone (Palaniraj & Jayaraman., 2011). Salts can also be used as precipitating agent in polyvalent forms(Pace & Righelato, 1981).

Biopolymer produced by X2 strain was recovered using microbial cell separation and alcohol precipitation technique. After removing the cells by centrifigutaion, chilled ethanol was added into the cell free fluid (CFF) in an optimized ratio of 3:1 (ethanol:CFF). The mixture was then kept at -18-20°C for efficient recovery of polymer.

Centrifugation and drying the pellet in dry heat oven separated the polymer from the cell free fluid + alcohol suspension. Figure 10 shows the recovered polymer. It was observed that this scheme of polymer purification led to 40% recovery of the polymer from the fermentation broth.

Conclusion

In order to reduce the production cost and to improve the competitive position of biopolymers, microbes are the best source leaving behind the plant-derived polysaccharides and synthetic polymers. Among various biopolymers, xanthan gum has gained importance in pharmaceuticals, cosmetics, cleaners, food, paints and various other industries.

In the present study 08 strains were isolated from the leaves of the infected plants. It was observed that different genera of bacterium including fecal contaminants and soil borne were present which showed similar lesions on leaves and other parts of plant. One (01) best biopolymer producing culture was selected to study the operational conditions for optimum polymer production i.e. agitation, temperature, pH, sugar concentration and incubation time. Highest production of biopolymer was found in the media containing 5% sucrose when culture was incubated at 30°C for 120 hrs at static condition.

The isolated strain X2 showed homology with the 16S ribosomal RNA gene of the partial sequence of *Stenotrophomonas sp. strain Ap10*(MK824562.1), *Bacterium strain BS1374* (MH890517.1), *Bacterium strain BS1240* (MK824428.1) and *Stenotrophomonas maltophilia strain IAE189* (MK414888.1). According to the originated timeline of the strain, the *Stenotrophomonas maltophilia* has capabilities to grow in an environment having different levels of oxygen, carbon dioxide and solar luminosities.

The isolated strain is novel of its kind and has not been exploited for biopolymer production so far at commercial level. The strain produces xanthan at low operational parameters, i.e. static or very low agitation with moderate temperature requirement. It grows and produce the polymer in condition easy to be managed at commercial settings. Further, studies to take up the strain at pilot scale production will validate it for commercial exploitations.

Declarations

- **Ethics approval and consent to participate:**

Not Applicable

- **Consent for publication:**

Not Applicable

- **Availability of data and materials:**

The rDNA sequence analyzed by ribotyping available in the GenBank repository, with an accession ID: MK422148 . weblink of dataset at GenBank

Whereas rest of all data generated or analyzed during this study are included in this published article [and its supplementary information files]

- **Competing interests:**

The authors declare that they have no competing interests

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- **Authors' contributions:**

Rashida Rahmat Zohra	Ideation and research study designing, DNA extraction, Ribotyping, Compilation of data, Result interpretation and supervision as Principal Investigator
Ayesha Siddiqui	Downstream processing of polymer
Mahnoor Saleem	Phylogenetic study, drafting and compilation of manuscript
Bushra Syed	Production parameters optimization
Heeba Shahid	Strain isolation and physic-chemical screening of the strains

All authors read and approved the final manuscript

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Figures

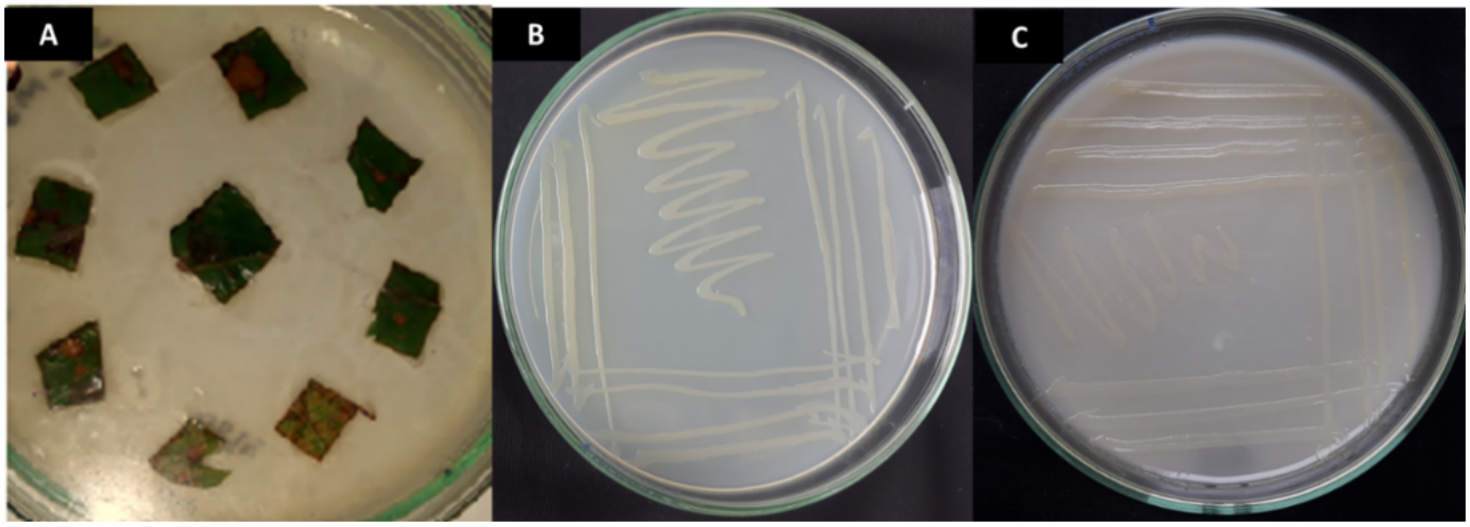

Figure 1

Infected leaves plating (A), Growth of Strenotrophomonas maltophilia strain X2 on nutrient agar (B) Growth of Strenotrophomonas maltophilia strain X2 on production agar plates.

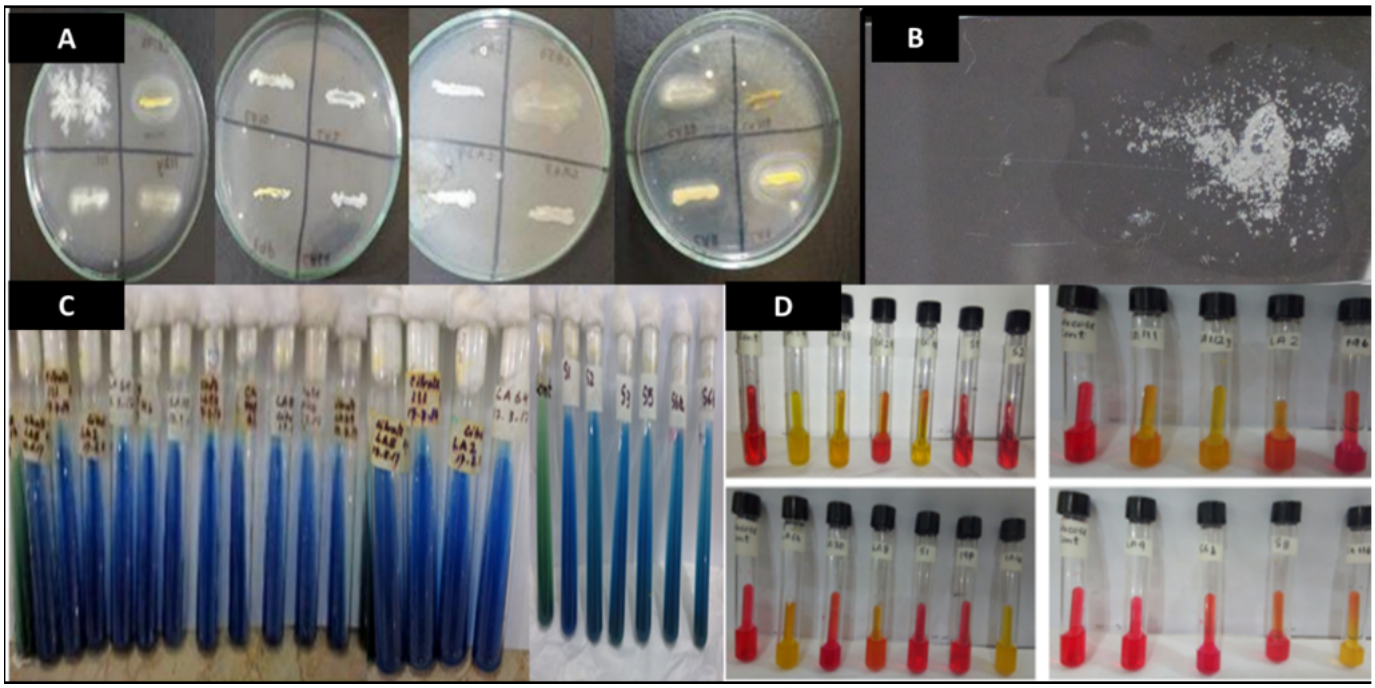

Figure 2

Plate assay of Tween 80 hydrolysis (A), Catalase test, Bubbles formation (B), Change in color indicating the utilization of citrate (C), Glucose fermentation indicated by change in color (D)

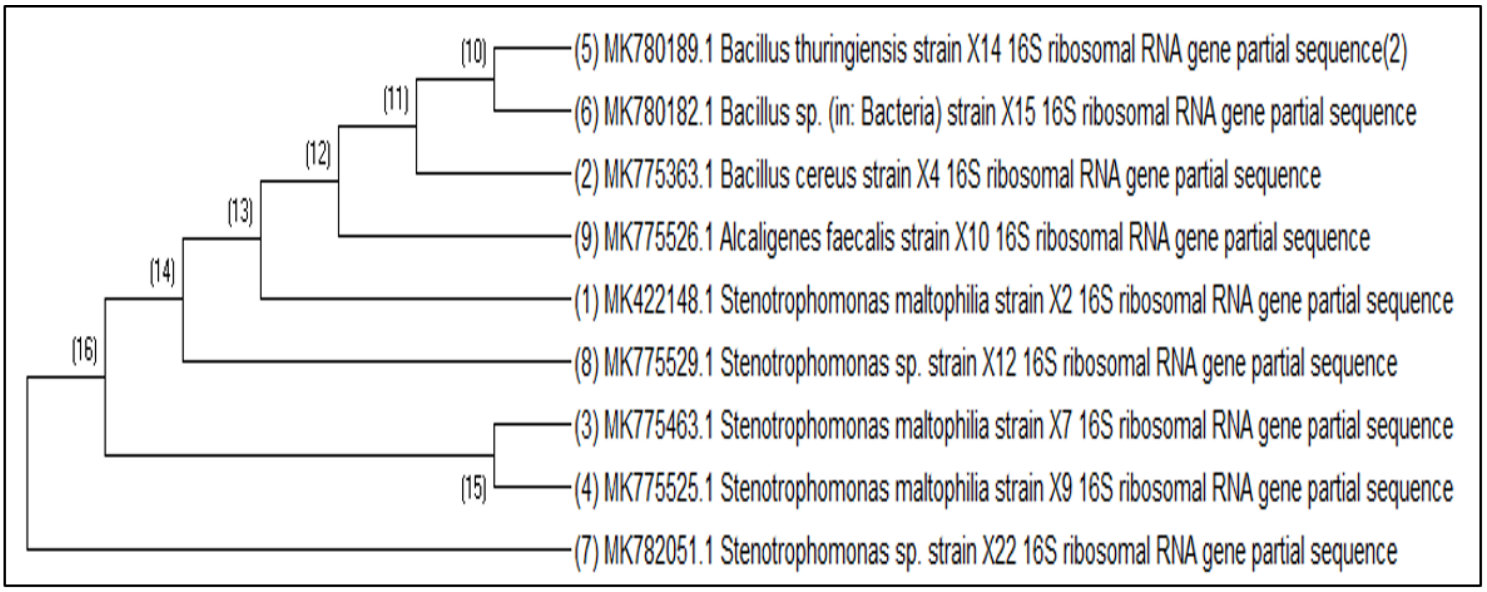

Figure 3

Phylogenetic tree of Stenotrophomonas maltophilia X2 (Accession# MK422148) (Kumar, et al., 2018).

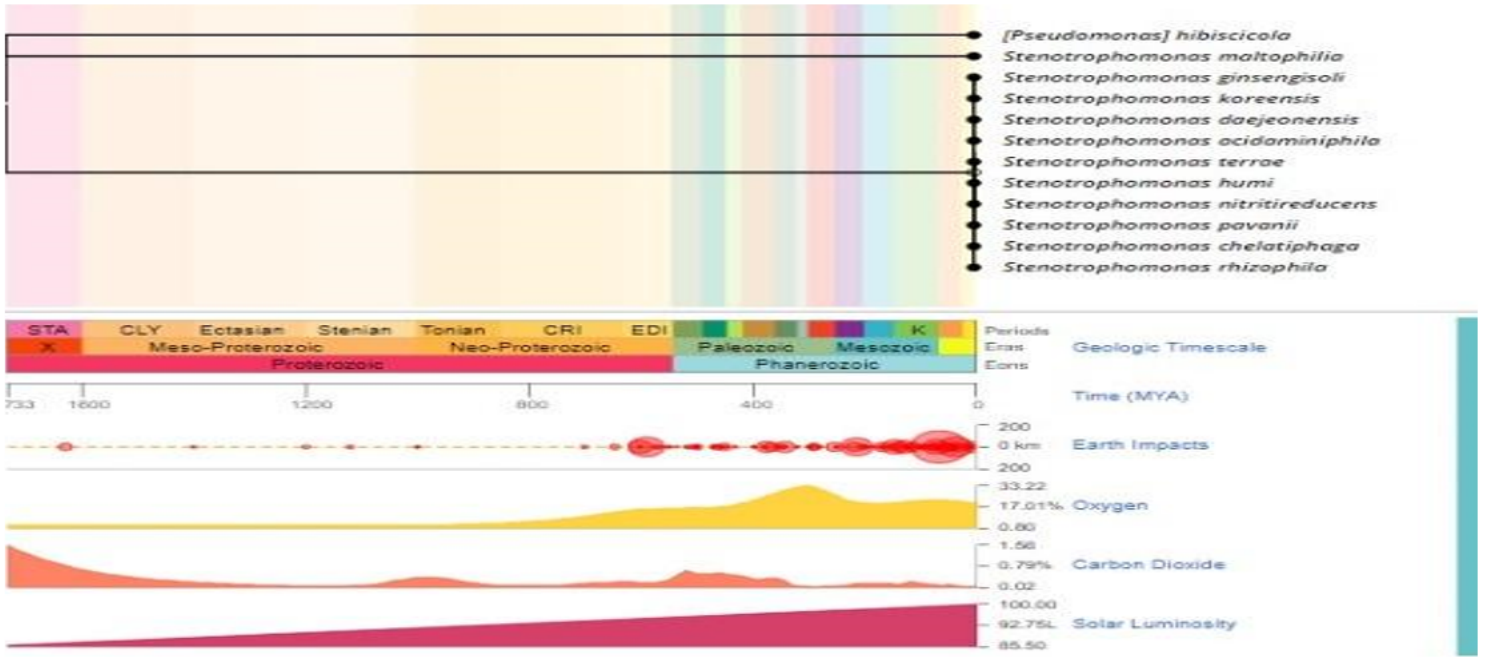

Figure 4

Timeline of Stenotrophomonas maltophilia. (Kumar, et al., 2018).

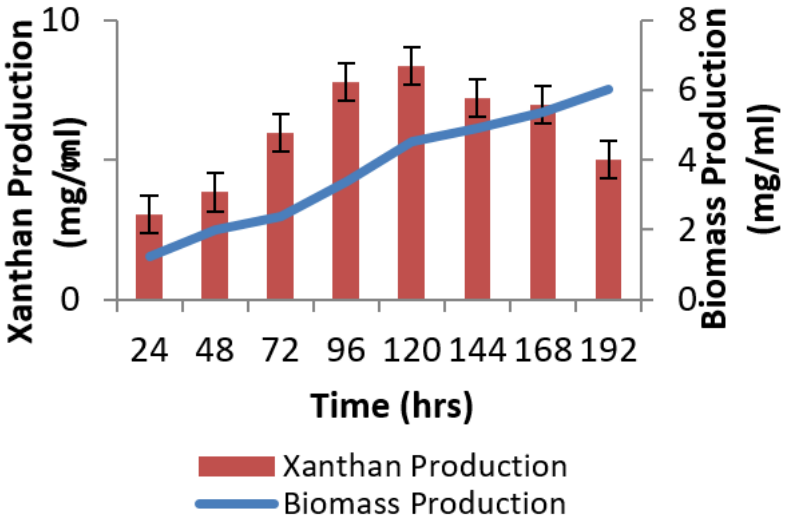

Figure 5

Production of xanthan at different time course

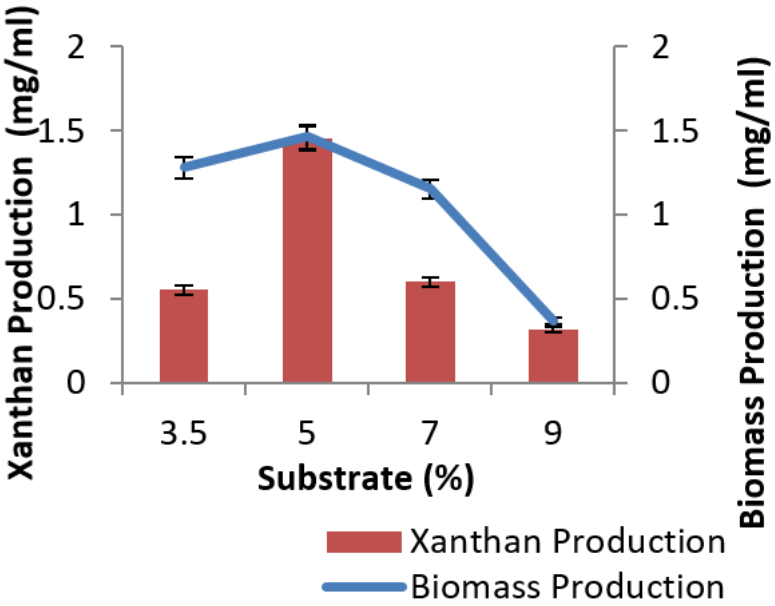

Figure 6

Production of xanthan at different substrate concentration

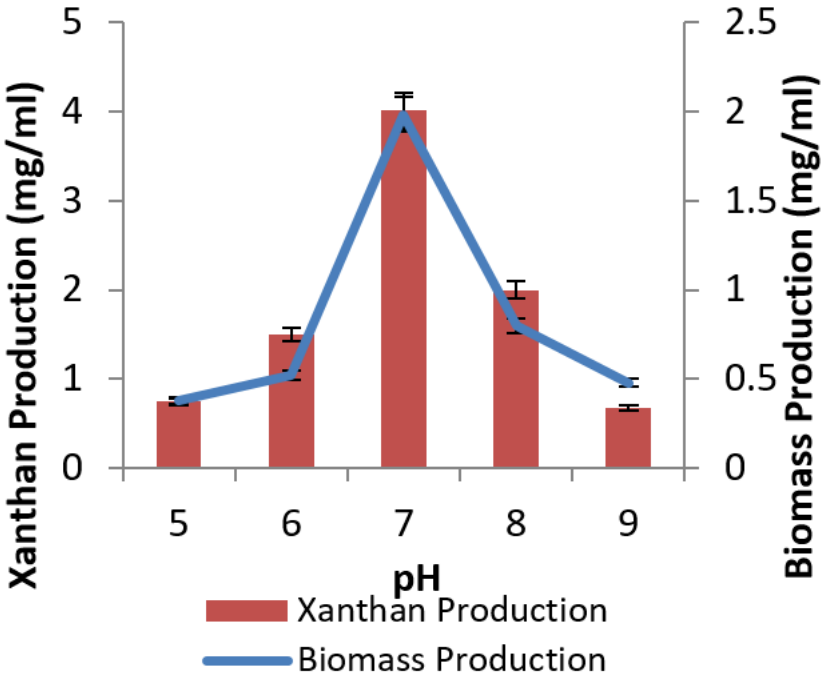

Figure 7

Production of xanthan at different pH

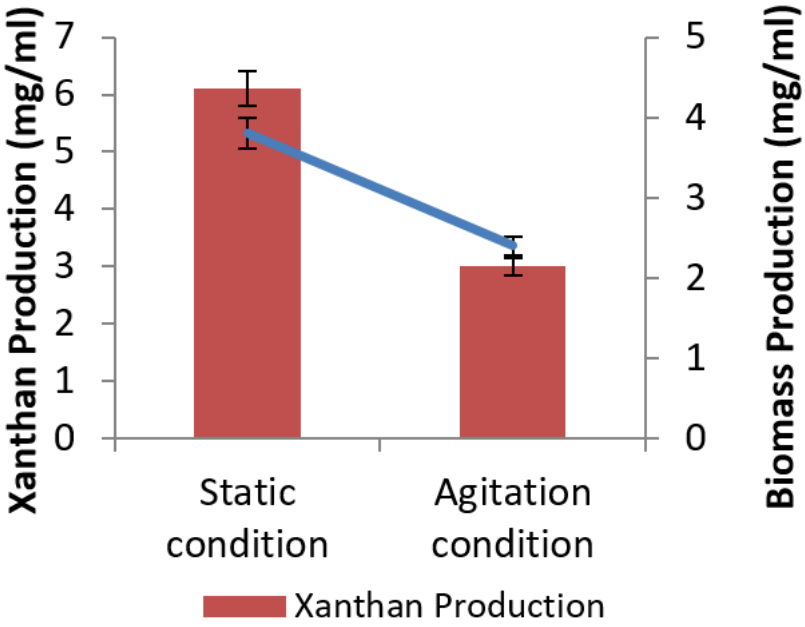

Figure 8

Production of xanthan at different growth conditions

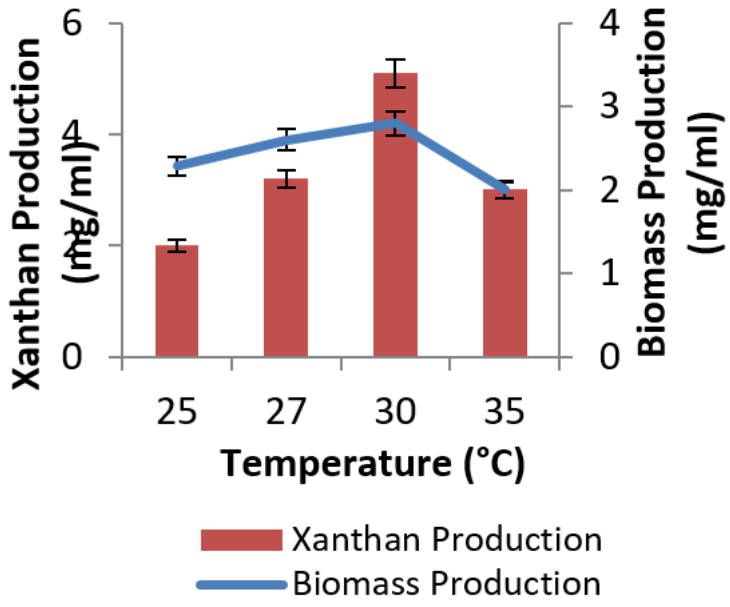

Figure 9

Production of xanthan at different temperatures

Figure 10

Extracted xanthan gum after purification

Supplementary Files

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