Vol. 19, Sup. 1 (2020) 213-225
Revista Mexicana de Ingeniería Química

Biosynthesis of fibrinolytic agent urokinase by *Enterococcus gallinarum* isolated from sardine

Biosíntesis del agente fibrinolítico uroquinasa por *Enterococcus gallinarum* aislado de sardine

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Received: May 2, 2020; Accepted: June 14, 2020

Abstract

Fibrinogen degradation by thrombin leads to the formation of fibrin clots in the blood which results in cardiovascular diseases due to which certain fibrinolytic agents are used to treat cardiovascular complications. Urokinase is being favored as a fibrinolytic agent due to its high fibrin specificity and fewer side effects. Therefore, the main objective of this research was the isolation of potent urokinase producing bacteria from soil, seawater, and fermented food samples. Thirty-five isolates that were capable of producing urokinase were isolated using sixty samples and subjected to secondary screening for estimation of fibrinolytic, caseinolytic and urokinase activity. Bacterial isolate QGA-20 that showed maximum casienolytic, fibrinolytic and urokinase activity i.e. 30.8± 0.03 U/ml/min, 52.5± 0.02 FU/ml/min and 41.22± 0.03 U/ml/min, respectively. In the rest of experiments, urokinase activity was determined in term of fibrinolytic activity because of cheap availability of substrate to estimate fibrinolytic activity. The maximum fibrinolytic agent producing strain was identified by 18s RNA sequencing as *Enterococcus gallinarum* which has never been reported before for urokinase production. Different physical and cultural parameters such as fermentation medium, incubation time, temperature, pH, carbon source, nitrogen source, inoculum size, and trace elements were optimized for submerged fermentation to synthesize fibrinolytic agent. Maximum production of fibrinolytic agent 81.3± 0.02 FU/ml/min was obtained with M-5 fermentation media at 96hrs of incubation, 37 ºC temperature, 7 pH, sucrose as carbon, and soya flour as a nitrogen source, 3% inoculum size and $CaCl_2$ as a trace element. In in vitro studies, urokinase enzyme was also applied for the disintegration of clot successfully. This study revealed a potent urokinase producer, reported first time, with appreciable clot lysis ability.

Keywords: Fibrinogen, thrombin, fibrin clots, in vitro, thrombosis.

Resumen

La degradación del fibrinógeno por la trombina conduce a la formación de coágulos de fibrina en la sangre que resultan en enfermedades cardiovasculares debido a las cuales se usan ciertos agentes fibrinolíticos para tratar complicaciones cardiovasculares. La uroquinasa está siendo favorecida como un agente fibrinolítico debido a su alta especificidad de fibrina y menos efectos secundarios. Por lo tanto, el objetivo principal de esta investigación fue el aislamiento de potentes bacterias productoras de uroquinasa del suelo, agua de mar y muestras de alimentos fermentados. Se aislaron treinta y cinco aislamientos que eran capaces de producir uroquinasa utilizando sesenta muestras y se sometieron a un cribado secundario para estimar la actividad fibrinolítica, caseinolítica y uroquinasa. Aislamiento bacteriano QGA-20 que mostró la máxima actividad casienolítica, fibrinolítica y uroquinasa, es decir, 30.8 ± 0.03 U/ml/min, 52.5 ± 0.02FU/ml/min and 41.22 ± 0.03U/ ml/min, respectivamente. En el resto de los experimentos, la actividad de la uroquinasa se determinó en términos de actividad fibrinolítica debido a la disponibilidad barata de sustrato para estimar la actividad fibrinolítica. La cepa máxima productora de agente fibrinolítico se identificó mediante la secuenciación de ARN de 18 s como *Enterococcus gallinarum*, que nunca antes se había informado para la producción de uroquinasa. Se optimizaron diferentes parámetros físicos y culturales como el medio de fermentación, el tiempo de incubación, la temperatura, el pH, la fuente de carbono, la fuente de nitrógeno, el tamaño del inóculo y los oligoelementos para la fermentación sumergida para sintetizar el agente fibrinolítico. La producción máxima de agente fibrinolítico se obtuvo 81.3 ± 0.02 FU / ml / min con medios de fermentación M-5 a las 96 horas de incubación, temperatura de 37 ºC, 7 pH, sacarosa como carbono y harina de soja como fuente de nitrógeno, 3% de tamaño de inóculo y $CaCl_2$ como un oligoelemento En estudios in vitro, la enzima uroquinasa también se aplicó para la desintegración del coágulo con éxito. Este estudio reveló un potente productor de uroquinasa, informado por primera vez, con una capacidad apreciable de lisis de coágulos.

Palabras clave: Fibrinógeno, trombina, coágulos de fibrina, in vitro, trombosis.

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https://doi.org/10.24275/rmiq/Bio1654
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Thrombosis is a condition in which fibrinogen degradation by thrombin leads to the formation of fibrin clots in the blood (Sharma *et al*., 2015). Embolism due to blood clots leads to death by causing a vascular blockage in the circulatory system (Kumaran *et al*., 2011). Cardiovascular complications due to thrombosis caused a high mortality rate in this world and this caused an alarming situation. For the degradation of blood clot plasminogen is present in blood as a zymogen (an inactive form of protein). The plasminogen is activated into plasmin to degrade the blood clot (Wen *et al*., 2018). There are several fibrinolytic enzymes including urokinase, nattokinase, and streptokinase that activate the plasminogen into plasmin so that the blood clot can be degraded (Moharam *et al*., 2019). Fibrinolytic enzymes have increased the survival rate of the patients by reducing the risk factor of thrombosis (Chen *et al*., 2011). Urokinase (E.C 3.4.99.6) is a fibrinolytic enzyme that is protease in nature and is a known plasminogen activator (Shakir *et al*., 2019). Plasminogen is activated into plasmin by the cleavage of the arginine-valine bond that results in the degradation of a blood clot. It is being used as a potent agent to treat cardiovascular diseases (Dong *et al*., 2016). It also helps in wound healing and activation of the complement system. Urokinase is being favored as a thrombolytic agent over other fibrinolytic enzymes due to its high fibrin specificity and less immunological effects as it is of human origin (Ebben *et al*., 2015).

Biotechnologists are finding ways so that these fibrinolytic enzymes can be produced economically which would have high fibrin specificity and low cost (Fajardo-Espinoza *et al*., 2019). Urokinase is currently being produced by fermentation technology and in vivo cell technology. Fermentation technology has led to the use of microbial sources like fungi (*Penicillium, Trichothecium, Asperigillus, Saccharomyces cerevisiae*), bacteria (*Pseudomonas, Halobacillus, E. Coli, B. subtilis, Enterococcos*), algae, and actinomycetes for the production of fibrinolytic enzymes (Ju *et al*., 2012). The bacterial source is preferred over fungal and yeast source due to its capacity of producing high yields of urokinase. Industrial production of fibrinolytic enzymes depends on the cultural conditions, physical parameters, and isolated strain (Gupta *et al*., 2002). Submerged as well as solid-state fermentation can be used to produce urokinase. However, submerged fermentation produces high yields of fibrinolytic enzymes as it enhances cell growth by increasing nutrient availability and the down streaming process is easy (Sharma *et al*., 2015). Optimization of cultural conditions (physical and nutritional parameters) is mainly focused to produce the maximum yield of microbial fibrinolytic protease (Bajaj *et al*., 2014). Media formulation is very important in the effective production of urokinase. Small changes in the media components lead to diverse results in the production and feasibility of enzymes and compel scientists to look for various types of media. Cost-effective media that can be used commercially for large scale production of urokinase is a major concern (Reddy *et al*., 2008). Urokinase is a known thrombolytic agent that can cure cardiovascular complications and have little or no side effects. In this report, we aim to study the isolation and screening of microflora that can produce urokinase. Optimization of physical and cultural parameters is also performed so that industrial-scale production of urokinase becomes feasible and economical. Furthermore, the efficacy of produced urokinase was assessed for blood clot disintegration.

2 Materials and methods

2.1 Chemical and reagents

Glucose, yeast extract, soy peptone, lactose, agar, casein, trichloroacetic acid, sodium hydroxide, human fibrinogen, thrombin, dibasic sodium phosphate, monobasic sodium phosphate, tris-chloride, sucrose, and magnesium sulphate were purchased from Merck, Sigma and Fischer scientific Ltd.

2.2 Isolation and primary screening of urokinase producing bacteria

Three different types of samples including soil (35), fermented food (15) such as Tuna, Sardine, Soyabean, Soya sauce, Hot sauce, Pickle, Mushroom, Chickpea, Olive, Corn, Red kidney beans, Tomato ketchup, Sweet corn and, seawater (Arabian sea Karachi) samples (10) were collected from Pakistan for the isolation of urokinase producing bacteria (Table 1). Nutrient agar was prepared and supplemented with 1% casein and human blood serum, each, after sterilization.

Table 1. Composition of fermentation media.

Serial no #	Media	Composition (100ml)	Reference
1.	M1	1g trypton, 0.5g yeast extract and 0.1g of NaCl	(Velumani, 2016)
2.	M2	1g caesin, 1g yeast extract, 0.5g peptone and 0.15g NaCl	(Vijayaraghavan P and Vincet P, 2014)
3.	M3	0.5g peptone, 0.5g caesin, 0.5g trypton, 0.5g beef extract, 0.5g yeast extract, 0.025g $MgSO_4$ and 0,5g sucrose	(Kumaran *et al.*, 2011)
4.	M4	0.5g peptone, 0.5g yeast extract, 1g casein and 0.15g NaCl	(Rovati *et al.,* 2009)
5.	M5	0.2g peptone, 1g casein, 1g yeast extract, 0.1g $CaCl_2$, 2g sucrose, 0.2g glucose and 0.2g potassium phosphate	(Seon *et al.*, 2002)
6.	M6	1g casein, 1g peptone, 1g trypton, 1g meat extract, 2g glucose, 0.5g yeast extract and 0.2 KH_2PO_4	(Smitha and pradeep, 2018)

The screened human blood serum used was obtained from blood bank of Services Institute of Medical Sciences (SIMS), Lahore Pakistan (Dubey *et al*., 2011). Soil and seawater samples were serially diluted (10^{-19}) and spread over the selective media plates. Fermented food samples were grinded and then homogenized in 0.2M sodium phosphate buffer (pH 7.5) to achieve a concentration of 1mg/ml and then 0.1ml of homogenized mixture for each sample was spread on the nutrient agar plates (Yoon *et al*., 2002).

2.3 Secondary Screening

Isolated bacterial strains were subjected to secondary screening for the estimation of casienolytic, fibrinolytic and urokinase activity. Fermentation media of pH 7 was prepared by mixing 0.2 g glucose, 1 g casein, 0.2 g potassium phosphate, and 2 g sucrose in 100 ml distilled water. Sterilized media was then inoculated with 1 ml inoculum (prepared by inoculating the nutrient broth 25ml with loop full of isolated bacterial strain and incubating at 37 ºC and 150 rpm overnight) and placed in shaking incubator (InnovaR 43 Incubator shaker series) at 37 ºC, 150rpm for 72hrs. After incubation, fermentation media was centrifuged (Model: EBA 20, Hettich, UK) for 15 min at 10,000 rpm. The pellet was discarded and the supernatant was used for further studies (Sharma *et al*., 2015).

2.4 Enzyme assay

2.4.1 Caseinolytic activity

The caseinolytic activity of urokinase was estimated by adding 50 μl of casein (1%), 50 μl enzyme supernatant, and 500 μl tris-HCl buffer (1 M; pH 7) in a test tube and incubating at 37 ºC in a shaking water bath (Model: WSB-30) for 3 hrs. The reaction was stopped by adding 600 μL of TCA (0.5 M) and the mixture was centrifuged for 10min at 10,000rpm. The pellet obtained was dissolved in NaOH solution (0.1 M) to which 1 ml of Folin's reagent was added. After 10min of incubation at room temperature, absorbance was recorded at 660nm using a spectrophotometer (Model: 7200 Aquarius, Cecil CE, United Kingdom). One casein degradation unit calculated as the amount of enzyme that degraded casein and release 1 μmol of tyrosine under given assay conditions (Martínez-Pérez *et al*., 2019).

Enzyme units are obtained by using the given formula (Abs = Absorbance, DF = Dilution Factor).

$$Unit = \frac{Absorbance\ (Test) - Absorbance\ (Control) \times DF}{0.01 \times time} \quad (1)$$

2.4.2 Fibrinolytic activity

Fibrinolytic activity was recorded using 0.4 ml of fibrinogen (0.1%) and 0.1 ml phosphate buffer (pH

7). The reaction mixture was placed at 37 ºC in a shaking water bath (Model: WSB-30) for about 15 min. Then 0.1 ml of thrombin (0.1% in 1 M phosphate buffer of pH 6) was added in the reaction mixture and subjected to incubation at 37 ºC for 10 min. After that, supernatant (0.1 ml) was added in the reaction mixture and it was incubated for an hour in a water bath at 37 ºC. The reaction was stopped by TCA (0.6 ml) and was centrifuged for 10 min at 13,000 rpm. The supernatant obtained was subjected to spectrophotometric analysis in the UV range at 275 nm (Rovati *et al*., 2010).

One Fibrin degradation unit was calculated as the amount of enzyme degraded fibrin and release one micromole of tyrosine and increase the absorbance to 0.01 per min (Wei *et al*., 2011). Enzyme units were obtained by using the given formula (Abs = Absorbance, DF = Dilution Factor).

$$Unit = \frac{Absorbance\ (Test) - Absorbance\ (Control) \times DF}{0.01 \times time} \quad (2)$$

2.4.3 Urokinase activity

Urokinase activity assay was performed using Activity Assay Kit (Fluorometric) (ab174098) using AMC-based peptide as substrate as it possess complementary binding sites for urokinase. After catalysis AMC was released and quantified by measuring its fluorescence at Ex/Em = 350/450 nm.

2.5 Strain identification

Best urokinase producing bacterial strain QGA-20 was sent to the Macrogen sequencing company, Korea for 16S rRNA sequencing. The genome sequence that had obtained was run in the BLAST. Homologs resulted from the Blast were then used in the Clustal W for constructing a phylogenetic tree to identify the bacterial strain QGA-20.

2.6 Optimization of physical and nutritional parameters

Different physical and nutritional parameters were optimized so that enhanced production of urokinase could be achieved. Six different types of fermentation media (the composition of media is given in Table 1) were prepared and subjected to submerged fermentation so that media with maximum enzyme activity could be optimized. Other factors such as incubation time (24-168 hrs), pH (5-9), temperature (27-42 ºC), Carbon source (mannitol, sucrose, fructose, starch, glucose, and lactose), Nitrogen source (soya flour, urea, trypton, and peptone), inoculum size (1-5%) and trace elements ($MgSO_4$, KH_2PO_4, NaCl, $CaCl_2$, K_2HPO_4, and NH_4SO_4) were optimized to achieve high yield of urokinase.

2.7 Application of urokinase (Clot lysis)

Human blood (1 ml) was taken in an Eppendorf that was placed in the water bath (37 ºC) for 15 min so that blood clots could be formed. The clot was then incubated with 0.1 ml produced enzyme and 0.1 ml of phosphate buffer (pH 7.0) for 3 hrs. The reaction was stopped by the addition of 0.3 ml TCA. The blood clot was observed for the disappearance. The control tube was run for the same reaction without the addition of the enzyme (Velumani, 2016).

2.8 Statistical Analysis

All the experiments were conducted in triplicates and the statistical analysis of the results was determined by Computer software, costat cs6204W.exe.

3 Results and discussion

3.1 Isolation and primary screening

Samples (soil, seawater, and fermented food) used for the isolation purpose were selected based on their high protein and nutrient contents (Kotb *et al*., 2016). Similar type of samples were also used by Mahajan *et al*. (2012) and Chen *et al*. (2013) with related approach for the isolation purpose. The selective medium used for the isolation of urokinase producing bacteria had blood serum in it as used by Dubey *et al*. (2011) in their research. For isolation and primary screening of urokinase producing bacteria soil samples (35), fermented food samples (15), as well as seawater samples (10), were collected from different areas of Pakistan. Thirty five bacterial isolates were found to have fibrinolytic activity and were screened further by submerged fermentation for estimation of fibrinolytic activity of enzyme produced (Table 2). Bacterial isolate QGA 20 was found with maximum fibrinolytic ability i.e. 52.5 ± 0.02 FU/ml/min.

Table 2. Primary and secondary screening of urokinase producing bacteria from soil, fermented food, and seawater samples.

Serial no #	Sample Label	Primary Screening	Bacterial Isolate	Urokinase activity (U/mL/min)
1	Nonar	+	QGA-1	10±0.05
2	Pakpattan	_	_	_
3	Jhatta	_	_	_
4	Wering	_	QGA-2	16±0.05
5	Bhatti, Lahore	+	QGA-3	29±0.05
6	Gohadpur	_	_	_
7	Lahore	+	QGA-4	35.2±0.02
8	Burewala	_	_	_
9	Kotli	_	_	_
10	Badomalhi	_	_	_
11	Mehmood boti	+	QGA-5	4±0.05
12	Gujranwala	+	QGA-6	18±0.03
13	Sargodha	_	_	_
14	Muridke	_	_	_
15	Ali pur	_	_	_
16	GCU, Lahore		QGA-7	12.5±0.05
17	Phulnagar		QGA-8	13.3±0.02
18	Sahiwal	_	_	_
19	Narang mandi	_	_	_
20	Lala Musa	_	_	_
21	Narrowal	+	QGA-9	30±0.03
22	Faisalabad	+	QGA-10	24±0.05
23	Kamoke	_	_	_
24	Muradpur	_	_	_

25	Gohad pur	_	_	_
26	Okara	_	_	_
27	Vehari	+	QGA-11	14±0.01
28	Sahiwal	+	QGA-12	15.2±0.05
29	Sheikhpura	_	_	_
30	Kasur	_	_	_
31	Chiniot	_	_	_
32	Gujrat	+	QGA-13	8.5±0.03
33	Bhalwal	+	QGA-14	19±0.05
34	Narrowal	+	QGA—15	6.8±0.02
35	Khanewal	_	_	_
36	Tuna (California garden fish)	+	QGA-16	42±0.03
37	Soyabean	+	QGA-17	31±0.03
38	Soya sauce	+	QGA-18	27±0.03
39	Light meat Tuna (Sultan flakes)	+	QGA-19	47.53±0.05
40	Sardine (2 diamond fish)	+	QGA-20	52.5±0.02
41	Mushrooms (Champignon Anmol brand)	+	QGA-21	34.93±0.01
42	Chickpea	+	QGA-22	13.9±0.02
43	Olive	+	QGA-23	29±0.03
44	Corn	+	QGA-24	31±0.02
45	Red kidney beans	+	QGA-25	34±0.06
46	Hot sauce	+	QGA-26	9±0.04
47	Tomato ketchup (National)	+	QGA-27	22±0.02
48	Mango pickle (National)	+	QGA-28	17±0.05
49	Sweet Corn	+	QGA-29	2±0.03

50	Tuna (Pepus food)	+	QGA-30	39.3±0.02
51	Arabian sea water 1	+	QGA-31	29.26±0.05
52	Arabian sea water 2	+	QGA-32	21.4±0.04
53	Arabian sea water 3	_	_	_
54	Arabian sea water 4	_	_	_
55	Arabian sea water 5	+	QGA-33	20.66±0.03
56	Arabian sea water 6	_	_	_
57	Arabian sea 7	+	QGA-34	19.93±0.02
58	Arabian sea 8	_	_	_
59	Arabian sea 9	_	_	_
60	Arabian sea 10	+	QGA-35	10.53±0.05

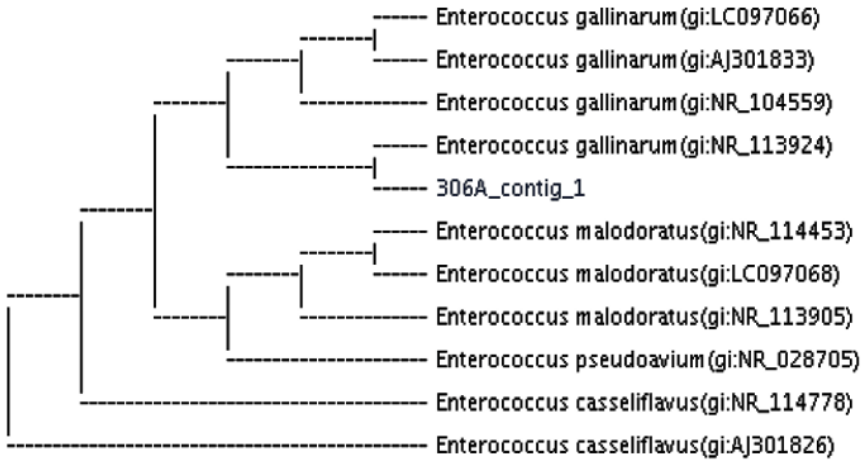

Fig. 1. The phylogenetic tree for the identification of bacterial isolate QGA-20.

3.2 Strain Identification

Maximum fibrinolytic agent producing bacterial strain QGA-20 was identified as *Enterococcus gallinarum*. The phylogenetic tree (Figure 1) depicts the relevance of query strain with the *Enterococcus gallinarum*. *Enterococcus faecalis* was reported by Yoon *et al*. (2002) as a producer of fibrinolytic protease and was identified similarly. Singh *et al*. (2014) identified *Enterococcus faecalis, Enterococcus faecium*, and *Pediococcus acidilactici* by similar method as fibrinolytic enzyme producers.

3.3 Enzyme production

Bacterial isolate (QGA-20), isolated from Sardine (fermented fish sample) showed fibrinolytic, caseinolytic and urokinase activity as 52.5 ± 0.02 FU/ml/min, 30.8 ± 0.03 U/ml/min and 41.22 ± 0.03 U/ml/min, respectively. There are many kinases available which possess both fibrinolytic and casienolytic activities. However, the specific reaction of enzyme produced in this study with urokinase specific substrate AMC-peptide showed the enzyme produced is urokinase with simultaneous fibrinolytic and caseinolytic activity as well.

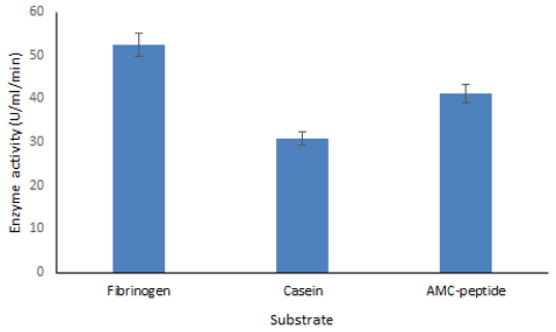

Fig. 2. Comparison of caseinolytic and fibrinolytic activity of bacterial isolate QGA-20.

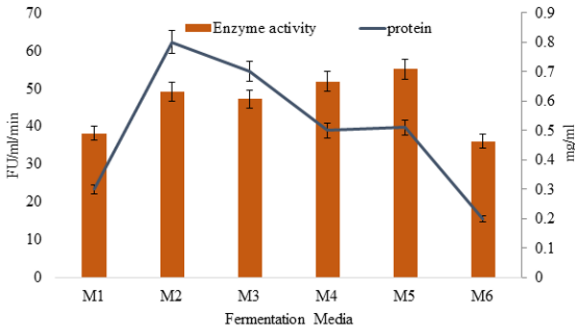

Fig. 3. Effect of different fermentation media on urokinase production by *E. gallinarum* QGA-20.

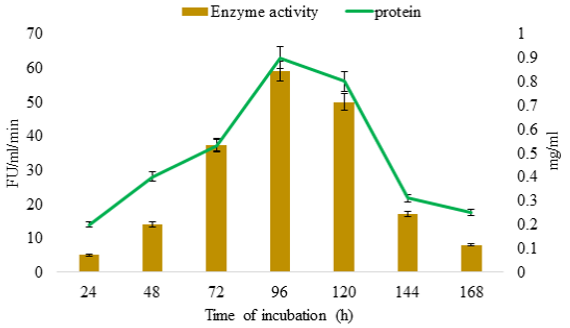

Fig. 4. Effect of incubation time on urokinase production by *E. gallinarum* QGA-20.

Moreover, figure 2 explains enzyme produced by bacterial isolate (QGA-20) resulted in fibrinolytic activity that was 1.75 and 1.27 folds higher than the caseinolytic and urokinase activity. Results of the current study are similar to those of Velumani (2016) who reported that the fibrinolytic activity (45 FU/ml/min) was 1.75 folds higher than that of caseinolytic activity (22 U/ml/min) for a microbial enzyme produced. Fibrinolytic enzymes are usually protease in nature and can degrade proteins such as casein and fibrinogen. High fibrinolytic activity indicates that urokinase is specific to fibrinogen degradation than that of casein or any other protein. To the best of our knowledge this is first ever report of urokinase production by *E. gallinarum* isolated from sardine fish. The enzyme activity in rest of the study was assessed in terms of fibrinolytic activity due to easy and cheap availability of substrate instead of using urokinase specific kit which is much costly. As both the substrates serves the same purpose there is no harm in using an alternative substrate.

3.4 Optimization of physical and nutritional parameters

Different types of fermentation media were used for maximum urokinase production. It was found that the highest enzyme activity (55.06 ± 0.05 FU/ml/min) was observed using the fermentation media M5 as shown in figure 3. However, the highest enzyme activity (59 ± 0.05 FU/ml/min) was recorded after 96hrs as shown in figure 4. Smitha and pradeep (2018) reported maximum activity of the fibrinolytic enzyme in minimal media after 96hrs by using *Bacillus altitudinis* S-CSR 0020. Bui *et al*. (2015) reported the maximum activity of fibrinolytic protease with enriched media after 48hrs by using *Bacillus weihenstephanensis*.

This contradiction to our results might be due to the different growth rates of bacterial strains used. Incubation temperature plays an important role in the growth of microbes as well as in enzyme production as it changes the physical properties of the cell membrane. The maximum enzyme yield (60.16 ± 0.05 FU/ml/min) was observed at incubation temperature 37 ºC as shown in figure 5. The results of the current study are comparable with the results of Moharam *et al*. (2019) who reported maximum enzyme activity at 37 ºC by using *Bacillus subtilis* as both the bacterial strain belongs to mesophilic temperature range. Sharma *et al*. (2015) reported maximum enzyme activity at 40 ºC by using *Citrobacter braaki*. These findings are contradictory to our results might be due to thermophilic nature of *Citrobacter braaki*.

Figure 6 indicates that the maximum fibrinolytic activity 65.46 ± 0.05 FU/ml/min was obtained when the pH of the fermentation media was set to neutral and these results are similar to those of Huy *et al*. (2016) who reported maximum enzyme activity with *Bacillus amyloliquefaciens* at neutral pH.

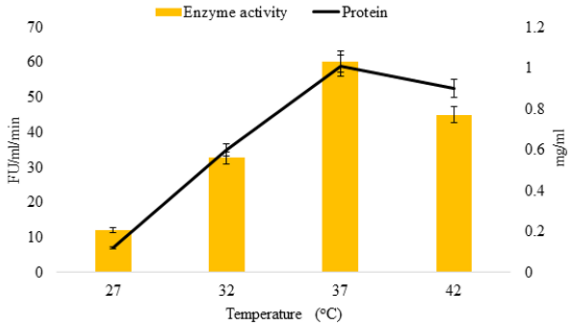

Fig. 5. Effect of incubation temperature on urokinase production by *E. gallinarum* QGA-20.

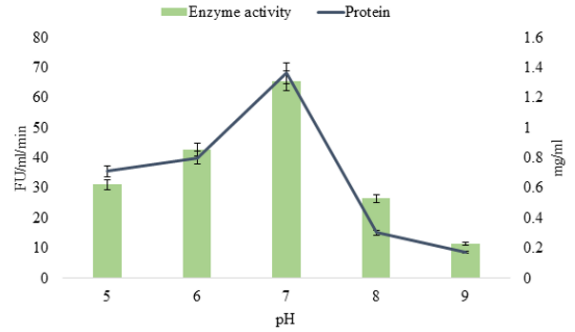

Fig. 6. Impact of initial pH of fermentation medium on urokinase production by *E. gallinarum* QGA-20.

The similarity in results is might be because isolation in both studies was carried out by using fermented food samples. Smitha and Pradeep (2018) reported maximum enzyme activity at basic pH by using *Bacillus altitudinis*. The difference is might be due to the fact that *Bacillus altitudinis* was isolated from the soil of slaughterhouse that was alkaline.

By optimizing carbon source, it was found that the maximum fibrinolytic activity (71 ± 0.05 FU/ml/min) was observed using sucrose as carbon source as illustrated in figure 7. Our results are comparable with the results of Vijayaraghavan and Vincent (2014) who reported maximum enzyme activity with *Paenibacillus* sp. when sucrose was used in the production media. The similarity in both studies might be due to the presence of certain chromosomes that favors the metabolization of sucrose.

Optimization of nitrogen source enhances the enzyme production. The highest enzyme activity (76.8 ± 0.05 FU/ml/min) was observed when the nitrogen source used in the production media was soya flour as shown in figure 8. Sharma *et al*. (2015) reported maximum enzyme activity with *Citrobacter braaki* when nitrogen source in the media was soya flour. Glutamine is the main component of soya flour that is used by microbes in certain metabolic pathways and it might be the reason for maximum enzyme activity. Pan *et al*. (2019) reported maximum enzyme activity with inorganic nitrogen source trypton by using *Bacillus subtilis* D21-8. Using soya flour as an organic nitrogen source in fermentation media compared to tryptone makes the production of urokinase more economical.

Maximum enzyme activity was recorded when the production media was inoculated with 3% inoculum as shown in figure 9. Wu *et al*. (2019) reported maximum enzyme activity with *Bacillus subtilis* WR350 by using 3% inoculum. At 1% and 2% inoculum size the bacterial cells were less in number and the nutrients level in the media was high and the growth rate was slow. At 4% and 5% inoculum size the bacterial cells were more in number and the nutrients level in media was less and the growth rate was affected. An optimum inoculum size 3% was required to stimulate the maximum bacterial growth so that high yields of fibrinolytic enzyme could be produced.

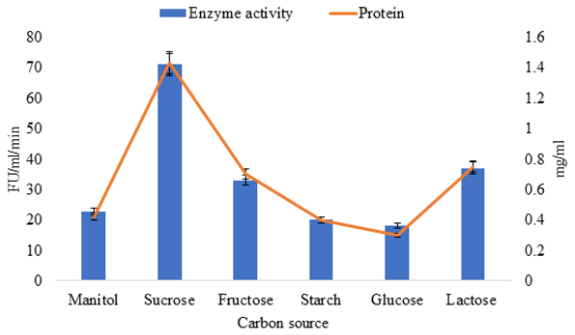

Fig. 7. Impact of various carbon source on urokinase production by *E. gallinarum* QGA-20.

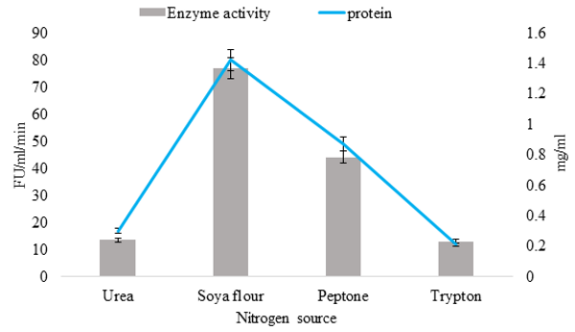

Fig. 8. Urokinase production by *E. gallinarum* QGA-20 using different nitrogen sources

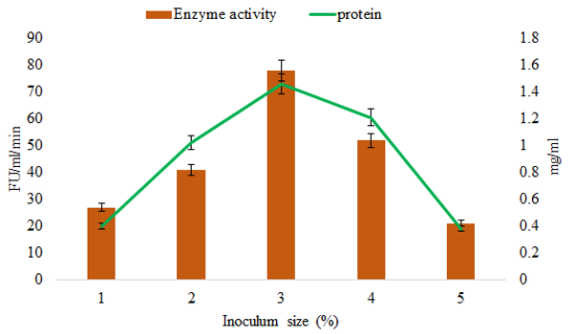

Fig. 9. Variation in inoculum size for improved urokinase production by *E. gallinarum* QGA-20.

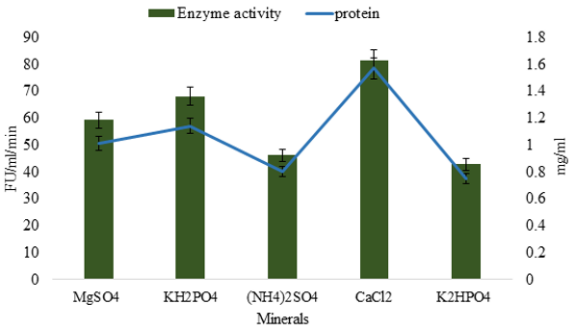

Fig. 10. Effect of various trace elements on urokinase production by *E. gallinarum* QGA-20.

Trace elements enhance the production of enzyme and it was found that by using $CaCl_2$ as trace element maximum enzyme activity (81.3 ± 0.05 FU/ml/min) was obtained as shown in figure 10. Mahajan *et al*. (2012) reported highest enzyme activity by using trace element $CaCl_2$ with *Bacillus subtilis* ICTF-1. It might be because enzymes require certain cofactors for their production so $CaCl_2$ found to be one of the best cofactors for fibrinolytic proteases.

3.5 Application of urokinase (Clot lysis)

Urokinase can be used as a thrombolytic agent to dissolve the blood clot. To analyze its application blood clot was dissolved in the laboratory in 3hrs as shown in figure 11. Dubey *et al*. (2011) reported the dissolution of blood clots using urokinase in 2hrs that was produced by *Pseudomonas* sp. Vijayaraghavan *et al*. (2016) generated blood clot by using animal blood and disintegrated it by streptokinase enzyme in 6hrs. Wang *et al*. (2008) dissolved the blood clot by the fibrinolytic enzyme that was produced by *Bacillus subtilis* LD-8547 in 24hrs.

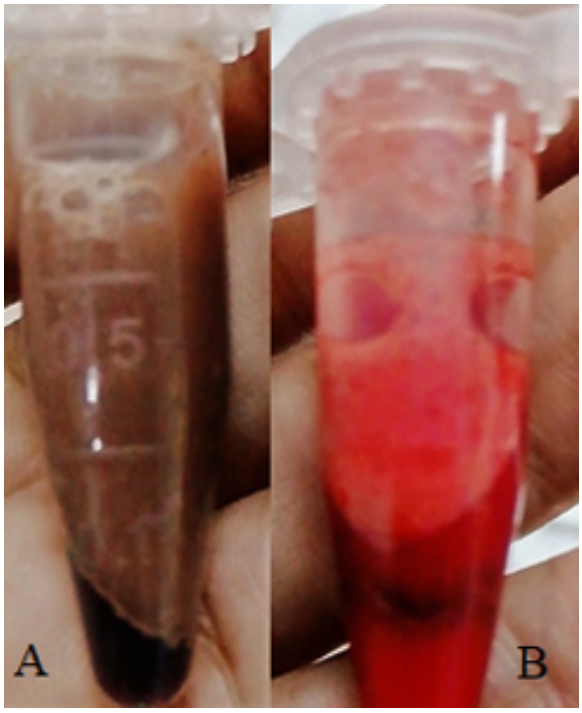

Fig. 11. Analysis of clot lysis ability of urokinases produced by *E. gallinarum* QGA-20: Tube A (Control: without enzyme) clot not dissolved, Tube B (Experimental: with enzyme) Clot completely dissolved.

This shows that urokinase produced in this study becomes highly promising thrombolytic agent as compared to other fibrinolytic enzymes for clot lysis purpose as it disintegrates the blood clot in less time without showing any severe complications.

Conclusions

The current study concludes fermented fish (Sardine) as the potent source for the isolation of urokinase producing bacteria. Optimization of physical and cultural parameters proved to have significant effect on enzyme synthesis and enhanced the production of urokinase by 1.75 folds with efficient clot lysis ability. This study will be useful for the production of fibrinolytic enzyme which can be used to treat cardiovascular complications.

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