JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANALYSIS OF NUTRITIONAL FACTORS INFLUENCING THE BIOSYNTHESIS OF AMYLOID BIOEMULSIFIER BE-AM1 APPLICABLE IN FOOD INDUSTRY

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https://doi.org/10.15414/jmbfs.3170

ARTICLE INFO

Received 22. 5. 2020
Revised 18. 3. 2021
Accepted 24. 3. 2021
Published 1. 8. 2021

Regular article

OPEN ACCESS

ABSTRACT

Thermostable glycoprotein amyloid bioemulsifier BE-AM1 was reported from *Solibacillus silvestris* AM1. *S. silvestris* could produce better BE-AM1 in 1% inoculum size and 18h inoculum age. The BE-AM1 production by *S. silvestris* AM1 has been seen to be unaffected by amending the production and growth medium (Zobel Marine medium) with Carbon and Nitrogen sources. The effects of 16 nutrient factors (from ZM medium) were analyzed statistically by one factor at a time, Plackett-Burman and Response surface methodologies. Among the nutritive factors, yeast extract and peptone were found to be significant, increasing the production quantity of BE-AM1. The time course of production of BE-AM1 was affected significantly by altering the release of the biomolecule from the surface of the bacterium. This will be the first study to analyse the effect of factors influencing the production of industrially important functional amyloid protein from bacterial source having potential in environmental remediation and microbial interaction.

Keywords: *Solibacillus*, bioemulsifiers, amyloid, Plackett-Burman, response surface method

INTRODUCTION

The high molecular weight amphipathic biosurfactants are also termed as Bioemulsifiers which act as capsular polymers that are released into environment by the producing strain during growth (**Amiriyan, *et al.* 2004**). Special attributes such as lower level of toxicity and higher degradability have naturally increased research interest and consumer demand for natural alternatives like the bioemulsifiers. The release of biomolecules from bacteria are influenced by many factors (physical and nutritional) as seen in the production of bioemulsifiers from *Corynebacterium* sp. *Pseudomonas* sp. and *Bacillus* sp. influenced by carbon sources like hydrophobic solvents (alkanes) and water soluble substrates respectively (**Amaral *et al.*, 2006**). Amyloid proteins are the filamentous proteins reported to be made up of amphipathic nature that aggregate to form fibres. The amyloids produced due to the mis-folding of the cellular proteins in mammalian tissues (leading to amyloid fibre formation) resulting in neurodegenerative diseases in hosts are termed pathological amyloids while their purposeful production in fungi and bacteria for specific physiological functions of the producing cell are known as functional amyloids **(Blanco, *et al.*, 2012; Nielsen *rt al.*, 2011**). The pathological amyloids (**Soreghan, *et al.*, 1994**) and functional amyloids **(Markande & Nerurkar, 2016a**) both have been reported for amphipathic nature and surfactant activities. A vast repertoire of biosurfactants have been reported with myriad of biomolecule contents and have the highest application food and allied industries (**Markande & Nerurkar, 2019**). Nutrient parameters influencing the production of biosurfactants having industrial applications especially in petroleum and environmental have been consistently reported **(Chai *et al.*, 2019; Hema *et al.*, 2019; Sharma & Pandey, 2020; Somoza-Coutiño *et al.*, 2020; Yaraguppi, *et al.*, 2020**). But the microbial amyloid bioemulsifiers have high applicability potential in food industry as they can be stable and can be extensively resistant to sterilization conditions.

Many polymeric carbohydrate based delivery systems (including cellulose, starch, pectin, chitosan, alginate, dextrin and cyclodextrins) are under study for utilization in food, pharmaceutical and other industries (**Fathi et al., 2014**). Self-assembling peptides having tailored structures possess extensive applications potential in nanotechnology **(Wei et al., 2017**). Amyloids like class I hydrophobins fall in the surface active agent category of amphipathic microbial functional amyloids that are extensively used in food industries (**Morris, et al., 2011**). Amyloids are potentially fundamental non-pathological protein folds being utilized by most living organisms (eukaryotic and prokaryotic) (**Fowler et al., 2006**).

Cao and Mezzenga, (2019) reviewed the various origins, formation conditions, chemical characters, potential applications and health implication of amyloid fibres from food proteins. Critical differences in packaging and assembly kinetics of pathological and functional amyloids dictates their potential uses in medicine and health-care (**Fowler et al., 2006**). The human microbiome associated amyloid proteins have the potential impact on amyloidogenesis of amyloidβ proteins in Alzheimer's disease and α-synnuclein proteins of Parkinsons disease (**Zhao and Lukiw, 2015; Sampson et al., 2020**). For therapeutic intervention of neurodegenerative disease, a way to alter is to influence the microbial partners and their products like amyloid proteins nutritionally (**Friedland and Chapman, 2017**).

Solibacillus silvestris AM1 was recently reported to be differing in biochemical and utilization properties from the type strain HR3-23T (**Markande, *et al.*, 2018**). *S. silvestris* AM1 was reported previously for the production of amyloid bioemulsifiers BE-AM1 having the ability to produce stable emulsions in aliphatic and aromatic hydrocarbons (**Markande *et al.*, 2013**). This bioemulsifiers was further reported to be thermostable amyloid protein (**Markande & Nerurkar, 2016a**) and capable of interacting with degraders for enhancement of remediation (**Markande & Nerurkar, 2016b**). BE-AM1 production by *S. silvestris* AM1 was consistent in pH and NaCl concentrations variations, but was affected by temperature variation due its influence on bacterial growth. BE-AM1 production was also seen at oligotrophic conditions mimicking natural environments (**Markande *et al.*, 2018**). Thus as the objective of present study were to elucidate the nutritive factors influencing the production of amyloid bioemulsifier BEAM1 using statistical methods like Plackett Burman and Response surface methodology. This will be the first report on the statistical analysis of nutrient influences on the production of bacterial functional amyloid having surfactant properties with potential applications in food industries.

MATERIAL AND METHODS

Strain and assays

Solibacillus silvestris AM1 (Accession number- MCC2096 at Microbial culture Collection Centre, NCCS, Pune, India) has been reported previously for production of amyloid bioemulsifiers- BE-AM1 only in Zobell Marine (ZM)

medium (composition given in Table 1) showing enhanced thermos-stability (**Markande & Nerurkar, 2016a; Markande *et al.*, 2013; Markande *et al.*, 2018**).

The production of amphipathic BE-AM1 was analysed by emulsification assay. Here, *S. silvestris* AM1 culture of 0.6 OD (A600) was grown in 50ml of ZM medium for 48h (at 30°C) and cell free supernatant was mixed with paraffin light oil (MERCK, Germany) at 1:1 using a homogenizer. The emulsification produced was analysed by calculating %EI as given by **Markande *et al.*, (2013**).

Table 1 Concentrations of the components used in OFAT and Plackett burman (PB) studies for the production of bioemulsifier by *S. silvestris* AM1

Sl. No.	Components	ZM medium	OFAT (g%)			PB (g%)	
			Low	Medium	High	High (+)	Low (-) in
1	**Peptone**	0.5	0.25	0.5	0.75	3	0.3
2	**Yeast extract**	0.1	0.05	0.1	0.15	1	0.1
3	**NaCl**	1.95	0.97	1.95	2.9	2.5	1.5
4	**$MgCl_2$**	0.88	0.44	0.88	1.32	1	0.25
5	**Na_2SO_4**	0.33	0.16	0.33	0.48	0.5	0.4
6	**$CaCl_2$**	0.18	0.09	0.18	0.27	0.4	0.2
7	**Fe-citrate**	0.01	-	0.01	-	0.01	0
8	**Na_2HCO_3**	0.016	-	0.016	-	0.016	0
9	**KCl**	0.055	-	0.055	-	0.2	0.05
10	**KBr**	0.008	-	0.008	-	0.04	0.008
11	**NaF**	0.00024	-	0.00024	-	0.0024	0
12	**$SrCl_2$**	0.0034	-	0.0034	-	0.0034	0
13	**H_3BO_3**	0.0022	-	0.0022	-	0.0022	0
14	**Na_2SiO_3**	0.0004	-	0.0004	-	0.004	0
15	**$(NH_4)_2NO_3$**	0.00016	-	0.00016	-	0.00016	0
16	**Na_2HPO_4**	0.0008	-	0.0008	-	0.0008	0

Bioemulsifier production

Inoculum size, inoculum age

Bacterial culture was grown as described for %EI. This overnight grown bacterial culture was pelleted and washed with phosphate buffered saline (PBS), adjusted to 0.6 OD (6.3×10^7 cells of *S. silvestris* AM1) and 1, 2 and 3% inoculums were added into sterile ZM broth (in triplicates) and incubated for 48h at 35°C. Similarly, a loop full of inoculums was added into sterile ZM medium and incubated at 35°C. After 6, 12, 18, 24 and 48h of incubation, aliquotes were removed, pelleted and adjusted to 0.6 OD (using PBS) and 1% was inoculated into 50ml sterile ZM medium in triplicates. After incubation, in both systems the culture was checked for OD and centrifuged for 10,000rpm for 10min. The cell-free supernatant was checked for %EI.

Analysis of influence of Galactose (1, 5, 10 and 15mg/ml) on BE-AM1 production by *S. silvestris* AM1

Galactose is a major carbohydrate that *S. silvestris* AM1 utilizes (**Markande *et al.*, 2018**). Growth and production of BE-AM1 in cell bound and cell free form was checked in different concentrations of Galactose (1, 5, 10, 15 mg/ml) in ZM salt solution. The inoculum was prepared as given for inoculum size experiments.

Statistical studies for medium component analysis

Madium: Standard Zobell-Marine (ZM 2116) medium was checked for the factors influencing the BE-AM1 production.

Table 2 Results of Plackett-Burman design for evaluating the components of ZM medium

Components	High (+) in g%	Low (-) in g%	E(xi)	t(Xi)	p-value	1-pvalue	Confidence
Peptone	3	0.3	23.09	2.82	0.04	0.96	96.29
Yeast extract	1	0.1	28.91	3.53	0.02	0.98	98.33
Nacl	2.5	1.5	-11.48	1.40	0.22	0.78	78.03
MgCl2	1	0.25	-14.37	1.75	0.14	0.86	86.03
Na2SO4	0.5	0.4	5.47	0.67	0.53	0.47	46.65
CaCl2	0.4	0.2	6.26	0.76	0.48	0.52	52.08
Fe-citrate	0.01	0	0.08	0.08	0.94	0.06	5.55
Na2HCO3	0.016	0	0.39	0.39	0.74	0.26	26.40
KCl	0.2	0.05	12.55	1.53	0.19	0.81	81.40
KBr	0.04	0.008	8.17	1.00	0.36	0.64	63.60
NaF	0.0024	0	0.17	0.17	0.88	0.12	12.18
SrCl2	0.0034	0	0.00	0.00	1.00	0.00	0.01
H3BO3	0.0022	0	0.18	0.18	0.88	0.12	12.49
Na2SiO3	0.004	0	0.61	0.61	0.61	0.39	39.44
NH4NO3	0.00016	0	0.85	0.85	0.48	0.52	51.53
Na2HPO4	0.0008	0	0.44	0.44	0.71	0.29	29.45

One factor at a time (OFAT)

Six macronutrients out of 16 total components of the ZM medium were studied by single factorial design. One independent macronutrient was taken with all the other components at their fixed level. Inoculum of 0.6 O.D was inoculated and the medium containing organism was incubated for 48hrs at 35°C. After 48hrs the medium was centrifuged at 10,000rpm for 15 min. The supernatant was further analyzed for emulsifying ability.

Plackett Burman design

To evaluate the relative importance of the components of the ZM medium, the Plackett-Burman experimental design (**Srinandan, *et al.*, 2010**) was used. After OFAT studies, each variable was tested at 2 levels, a higher (+) and a lower (-). The 16 medium components were used as independent variables and three dummy variables were set to estimate the experimental error supplementary table 2. The rows in supplementary table 2 shows experimental trials and each column represent different variables. The medium components in design were as given in table 1. The results were tested in triplicates.

The effects of each variable was determined with the following equation

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

where, Exi is the concentration effect of the tested variable, Mi+ and Mi- are the emulsification indices from the trials where the variable (Xi) measured was present at high and low concentrations respectively and N is the number of trials (**Srinandan *et al.*, 2010**). Since the objective of this study is to evaluate the relative effect of each variable, a significance level of less than 0.2 is acceptable. Significance of the triplicate values of each trial was calculated using Prism 5.0 software.

Response surface methodology

Response surface methodology using Box-Behnken design was used to estimate the nutritional factors important in BE-AM1 production using central composite design. The medium components used in the design were A: peptone (3, 1.65 and 0.3g%),B: yeast extract (0.1, 0.55 and 1g%), C: $MgCl_2$ (0.25, 0.625 and1g%) and D: KCl (0.05, 0.125and 0.2g%). The behavior of the system was explained by,

$$Y = \beta_o + \Sigma\beta ixi + \Sigma\beta ijxixj + \Sigma\beta iixi^2$$

where, Y is the predicted response, β_o is offset term, βi is a linear offset, βii is squared offset and βij is interaction effect. xi dimensionless coded value of Xi. This equation was further solved by using the software Design-Expert (version

8.0, Stat-Ease, Minneapolis, MN, USA) and the responses of the dependent variables were estimated.

Critical micellar dilution

Critical micellar dilution (CMD) indicates a minimum biosurfactant/bioemulsifier required for effective micelle formation, and is a measure of bioemulsifier concentration and its production. By diluting the crude amyloid BE-AM1 produced in ZM and AM1 medium (newly formulated combination of ZM medium components) with distilled water, the %EI is measured. The dilution at which, %EI starts to fall abruptly is the critical micellar dilution (CMD), where it is proportional to the amount of biosurfactant/bioemulsifier present in the sample (**Markande *et al*., 2013**).

Influence of Peptone on production of BE-AM1 by *S. silvestris* AM1

To check the effect of peptone (in low concentrations amended in ZM medium) on the production of amyloid BE-AM1 by *S. silvestris* AM1. The bacterium was inoculated in synthetic marine salt (ZM salts) solution containing different concentrations of protein (0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, 10.0, 12.5 mg/ml Peptone).

Time course production of amyloid BE-AM1

ZM medium and AM1 medium (50 ml each) were inoculated with 6.3×10^7 (0.6OD) *S. silvestris* AM1 cells. The system was kept for incubation at 30°C. Every hour, 2ml aliquots were taken out and centrifuged at 10,000 RPM for 10min. The cell pallet was given two washes with phosphate buffered saline (PBS; 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4 at pH 7.3) and 2ml cell suspension was made in PBS. Centrifugation supernatant and cell suspension were checked for %EI (E_{24}).
Time course of BE-AM1 production by *S. silvestris* AM1 in oligotrophic conditions of protein presence was checked by growing it in presence of 0.5 mg/ml Peptone in ZM salt solution as given before at a sampling interval of every two hours from the point of inoculation.

RESULTS AND DISCUSSION

Nutritional factors affecting amyloid BE-AM1 production

The amyloids are extremely resistant proteins capable of withstanding physical (thermal), chemical and enzymatic denaturation **(Gebbink, *et al*., 2005; Nielsen *et al*., 2011**). These are extensively seen to be produced by microorganisms like fungi (**Blanco *et al*., 2012**), actinomycetes (**Hammer, *et al*., 2008**) and bacteria (**Dueholm, *et al*., 2013**) with specific functional and physiological intent (termed Functional amyloids) helping in microbial adhesion (**Larsen *et al*., 2007; Lipke *et al*., 2012; Nielsen *et al*., 2011**) capable of deciding microbial pathogenicity **(Gebbink et al., 2005; Schwartz and Boles, 2013)**. Many microbial biosurfactants have been reported for applications in food industries (**Markande & Nerurkar, 2019**).
The amyloid fibre formation during food processing are considered rare. But can get triggered into cross-reactive amyloids in gastric conditions or may provide raw materials for bacterial functional amyloids further enhancing human pathological amyloidogenesis (**Lambrecht et al., 2019**). Amyloidogenesis can be evolutionarily conserved ancient quaternary protein structure contributing to normal cell and tissue physiology (**Fowler et al., 2006**). **Friedland and Chapman (2017)** termed microbiota associated proteopathyand subsequent neuroinflamation as “mapranosis”.
Although different from pathological amyloids, the food protein amyloids have uses as advanced biomaterials in medicine and tissue engineering, nanotechnology, environmental science, material sciences and in food sciences(**Cao and Mezzenga, 2019**). Food amyloids from whey, kidney bean, soy bean and egg white exhibited resistance to enzymatic digestion like Proteinase K treatment or Pancreatin (**Lassé et al., 2016**). Amyloid proteins have immense applications as effective gelling agents due to their functional surface chemistry (**Lassé et al., 2016**).
Although the southeast coast of India was relatively stable for last 50 years, their characteristics have been significantly changed by Indian ocean tsunami (26^{th} December 2004) **(Senthilnathan, *et al*., 2012**). The Vellar estuary (Tamil Nadu, India) present in this area has been extensively occupied by shrimp and prawn cultivation - enriching the water in the estuary with protein content. Here (Vellar estuary)- physical (temperature, pH and salinity) and chemical factors (carbon, nitrogen and phosphorus content) were reported to be undergoing distinct seasonal fluctuations and changes with respect to the textural type of the sediment **(Sundaramanickam, *et al*., 2008**).
The type strain of *Solibacillus silvestrris* was isolated from German forest soil (**Rheims, *et al*., 1999)** and was also reported for antineoplastic activities (**Pettit *et al.*, 2009**). *S. silvestris* AM1 is an estuarine isolate from Perangipettai, Tamil Nadu, India at Vellar estuary and differed from type strain in utilization studies and its ability to form amyloid BE-AM1 (**Markande *et al.*, 2018**).

It was reported to be able to produce amyloid BE-AM1 only in ZM medium but differed in many utilization aspects from type strain HR3-23^T (**Markande *et al*., 2013; Markande *et al*., 2018; Rheims, *et al*., 1999**). As given in materials and methods, thirteen macro and micronutrients as salts in ZM medium make up around 3.4%. Addition of NaCl to the medium in g% increases the salt content to 3.55 to 8.55% respectively starting from 0% amendment (**Markande *et al.*, 2018**).
Inoculum age and inoculum size are the two factors usually known to affect the production of microbial surfactants like amyloid BE-AM1s. The size and age of the bacteria being added for production of a specific compound are important factors in deciding final yield of the product. 1ml of 0.6 O.D. of *S. silvestris* AM1 gave a better result than higher cell number. Similarly, addition of 18h grown culture resulted in higher amyloid BE-AM1 production.

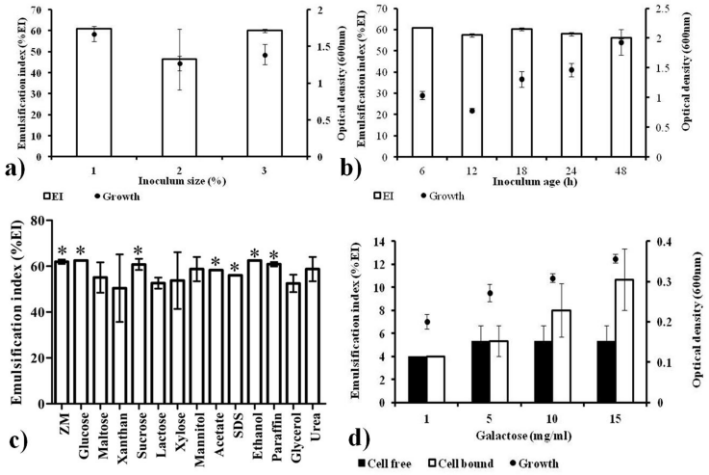

Figure 1 Effect of inoculum size (a) and inoculum age (b) on emulsification activity of *S. silvestris* AM1. (n=3; p values, Δ=p<0.001 and *=p<0.05). (c) Emulsification index (%EI) of *S. silvestris* AM1 grown in ZM medium and its amendments with given sources (*=p<0.001*)*; (d) Effects of Galactose on growth and emulsification properties of *S. silvestris* AM1 (n=3; p-values: *=p < 0.01).

Among the carbohydrates, *S. silvestris* AM1 utilizes galactose prominently (Table S1). As given in figure. 1d. Galactose, used as a sole carbon source, could promote the growth of *S. silvestris* AM1 with increased galactose concentration (0.2 to 0.36 O.D.), but gave negligible BE-AM1 production (2 - 5.3% EI). *S.silvestris* AM1 has a limited ability to utilize the carbohydrates resulting in inability of glucose, sucrose and other sugars in increasing the amyloid BE-AM1 production. Addition of carbon sources to ZM medium did not induce amyloid BE-AM1 production. The lower bacterial growth in presence of some nutrient sources resulted in lower amyloid BE-AM1 production. Galactose being the major carbohydrate present in amyloid BE-AM1 produced (**Markande *et al*., 2013**), it was checked for increasing the amyloid BE-AM1 production. Presence of galactose marginally increased emulsification process. Human Pmel17 protein synthesizes tissue amyloid proteins in tight regulation to avoid toxicity which further has important roles in pigment production and activation of hemostatic system (**Fowler et al., 2007**). In pathological amyloidogenesis, salt ions are known to influence the course of misfolding of protein, its aggregation and amyloid formation (**Dogra, et al., 2020**).

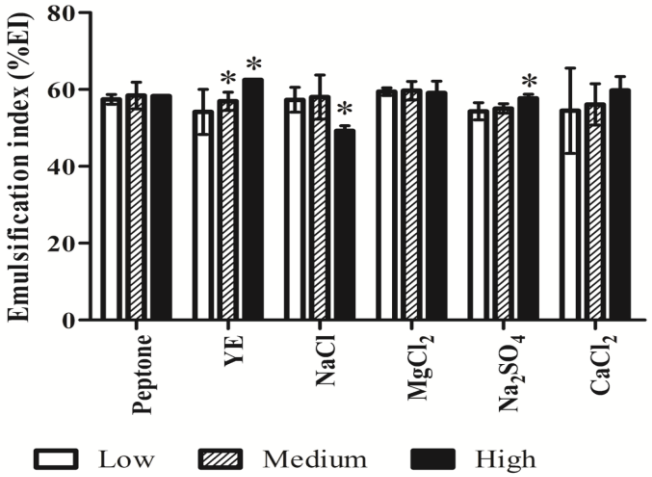

Figure 2 Effect of changing one factor at a time (OFAT) of the macronutrients from ZM medium on emulsification of *S. silvestris* AM1. (p-value, *<0.001).

The amyloid BE-AM1 produced in the range of peptone and $MgCl_2$ used in OFAT studies does not significantly affect the amyloid bioemulsifier production (pValue=0.4 and 0.9 respectively). 57-59% EI was produced in presence of the two compounds. Changes in the concentration of YE affect the production of amyloid BE-AM1 significantly (pValue<0.02) with a range of 54-64% EI. NaCl, Na_2SO_4 and $CaCl_2$ showed varied effect on the production of amyloid BE-AM1 with the EI range between 49-59%. As reported by many, the one factor at a time (OFAT) approach is time consuming but can be used for identifying critical components of the medium under study (**Sivapathasekaran, *et al.*, 2010; Srinandan *et al.*, 2010**). According to OFAT studies, the ranges of peptone and $MgCl_2$ concentration taken have little significance on amyloid BE-AM1 production while yeast extract was found to be a significant factor. Using Plackett-burman design, the medium components giving highest emulsification (%EI = 68% in trial 9) was achieved. Although the increase in emulsification is just for 10%, from calculations, it can be stated that yeast extract is a significant factor which was also seen in OFAT studies (figure 2).

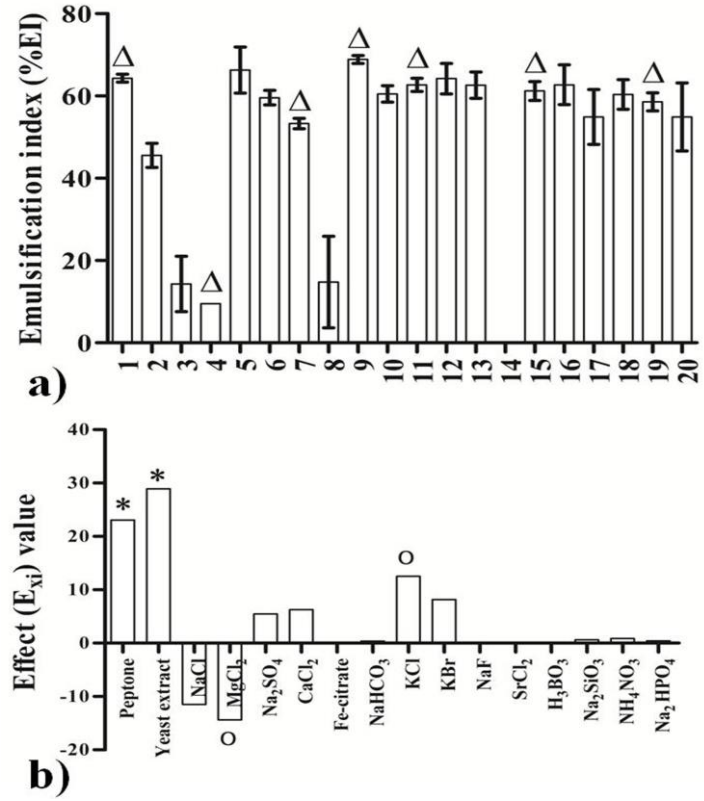

Figure 3 (a) The emulsification in presence of the 20 trial media of Plackett-Burman design (p-value, Δ<0.001); (b) Effects calculated for each of the components of ZM medium (p-value, *<0.05, O<0.2).

When Plackett burman design was checked for effect of each constituents for amyloid BE-AM1 production (figure. 3b), effects of peptone, yeast extract were highest in the positive side with KCl, while MgCl2 showed highest negative effect for amyloid BE-AM1 production. Although it is known that less than 5% variation in the confidence of the matrix is sufficient, the amyloid BE-AM1 studies with matrix analysis of Plackett-Burman design is robust and it rarely shows the probability of observations arising more than 20% of the confidence is rarely due to chance. Hence $MgCl_2$ and KCl, both giving significance p-value<0.2 were selected for further studies.

Industrial processing may induce food protein to re-fold to form effective amyloid monomers capable of forming fibres as in case of boiling of hen egg white (**Monge-Morera et al., 2020**). Manipulation of protein processing conditions for core regions of food protein which fold into amyloids and further promote sequential and ordered aggregation can enhance and control food functionality. Thus amyloid fibril formation in food protein can help tailor aggregation and fibril-techno functionality (**Jansen et al., 2019**).

Sundaramanickam *et al.*, (2008) reported the rapid increase of shrimp farms (42 till 1995, covering an area of 150ha.) along the vellar estuary. The feed pellet given to the shrimps have become the major source of eutrophication in the estuary. This release of used water from the shrimp farms into the backwaters, estuaries and mangroves affecting the biological communities and has become a major concern for environmentalists **(Sundaramanickam *et al.*, 2008)**. To study the significant component of the Zobell-marine medium, Plackett-Burman design and Response surface methodology were used. The amyloid BE-AM1 production, quantified as emulsification index (%EI) from the cell-free supernatant of the media trials are given in table 2. As shown in figure. 3a, trial 9 showed highest EI of 68.9% and trial 5 showed 66.3% EI. Trials 1, 11, 12, 13, 15, 16 and 18 gave 60-64% EI, nearer to ZM medium's 62.5% EI. Trial 4 gave lowest EI of 9.5%. The four components of ZM medium shortlisted for their significant positive influence in production of amyloid BE-AM1 by *S. silvestris* AM1 were analyzed using Box-Behnken design in Design Expert 8.0 software. Similar to Plackett-Burman design, peptone and yeast extract were found to be the most significant factors among the four components shortlisted from Zobell marine medium. Although the four factors did not interact for amyloid BE-AM1 production, they interacted significantly for surface tension reduction (data not shown). The ZM medium and its four components making up AM1 medium were checked for amount of amyloid BE-AM1 release and were found to be more as the emulsification activity is seen even after 1000times dilution.

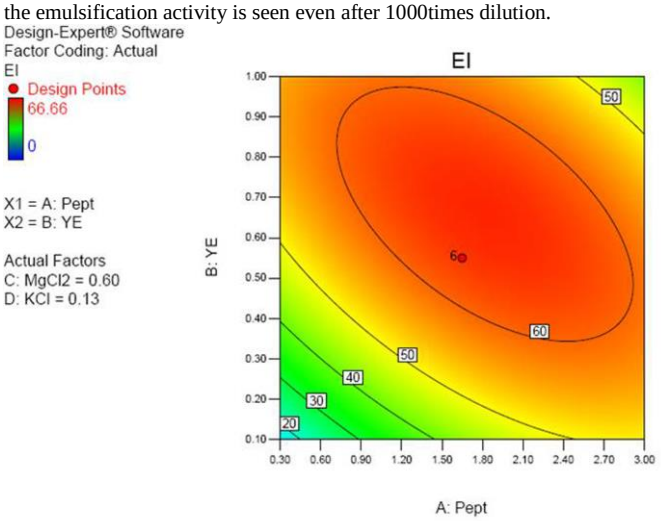

Figure 4 Contour graph of analysis of Emulsification index by Design Expert software version 8.0 of StatEase

Effects (Exi) of the components of ZM medium were calculated as given before and are given in figure. 3b. Positive Exi value of the variable is considered to be positively influencing *S. silvestris* Am1 in higher BE-AM1 production among the ranges of the concentrations taken, and negative result shows its positive influence in lower concentrations. Peptone and YE influenced significantly on the production of amyloid BE-AM1 (p<0.05) while $MgCl_2$ and KCl influence with lower significance (p<0.2). As shown in figure. 3b peptone, YE and KCl showed positive Exi values. $MgCl_2$ showed negative Exi value showing its influence in enhancing the production of amyloid BE-AM1 by *S. silvestris* in its lower concentrations taken for this test (table 2).

Design Expert version 8.0 of StatEase was used to build Box-Behnken design and to analyze the results obtained. These components were studied for their interactions and use of this model for higher production of amyloid BE-AM1. After the analysis, the response yielded a linear model as there was no interaction seen among the components for amyloid BE-AM1 production. Peptone and yeast extract were found to be most significant components influencing the production with p-values of <0.0001 and 0.0044 respectively giving the hot zone as given in figure. 4. Since F-value of model was found to be 10.22, there is only a 0.01% chance that a 'model F-value' this large could occur due to noise. The lack of fit value for the model was not significant (p=0.7494). Thus, there is a 74.94% chance that a lack of fit F-value of 0.68 could occur due to noise or pure error. Thus the model and its terms were found to be significant.

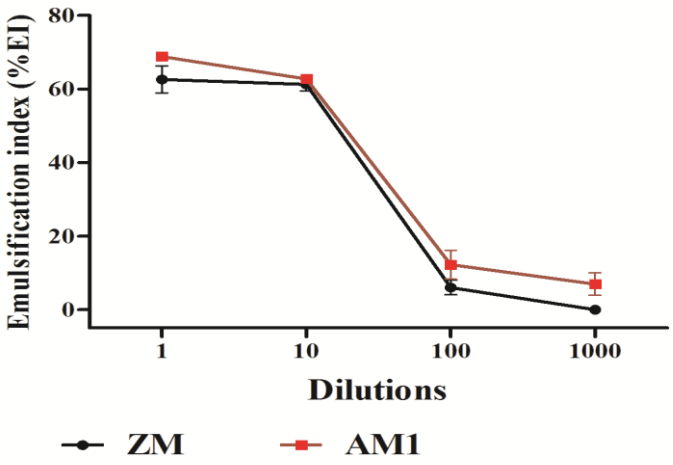

Figure 5 Critical micellar dilution (CMD) of bioemulsifier produced in ZM medium and optimized AM1 medium

The important nutritional factors shown by Placket-Burman design for amyloid BE-AM1 production were compared with ZM by checking for CMD in the two media. Production of amyloid BE-AM1 was unaffected in both the media with CMD showing slight defferences (figure. 5). In presence of minimal nutritional factors (in medium AM1), *S. silvestris* AM1 produced 68% EI as compared to 62% EI in ZM medium, retaining 9.7% more emulsification activity with after 1000 times dilution. Amyloid protein formation was shown to be enhanced in presence of hydrolysed proteins (**Lambrecht et al., 2019**).

Time course of BE-AM1 release

At 12h, *S. silvestris* AM1 reaches stationary growth and produced amyloid BE-AM1 without hydrocarbon-induction. The bacterium exhibits cell-bound emulsification activity (%EI) after 6h growth (mid-log phase) while cell free emulsification activity can be detected after 16h, when the culture's growth is in a stationary phase (data not shown). Emulsan is known to accumulate on the cell-surface of *A. calcoaceticus* RAG-1 as a mini-capsule during logarithmic phase of growth and like many other extracellular polysaccharides, it is produced after the cells reach stationary phase, similar results were observed in case of amyloid BE-AM1 production in *S. silvestris* AM1. Bacterial amyloids have been reported for their amphipathic nature and surface adherence properties (**Markande & Nerurkar, 2016a**). As the lesser significant components namely of ZM medium were eliminated in the AM1 medium, the amyloid BE-AM1 release by the bacterium is changed effectively. Release of cell-free amyloid BE-AM1 is decreased by 8h (figure. 6), while the lag phase of the bacterium is increased by 2h. The cell bound emulsifier is produced at higher amounts in oligotrophic conditions (figure. 6). In natural conditions, with competition for nutrients, bacteria produce more cell-bound amyloid BE-AM1 than cell free.

Presence of optimum environmental (including intrinsic and extrinsic) and nutritive factors like aminoacids and peptides prone to amyloidogenesis can lead to food protein conversion to amyloid fibrils. This has been extensively studied for egg, cereals, milk and legume proteins (**Jansens et al., 2019; Lambrecht et al., 2019**).

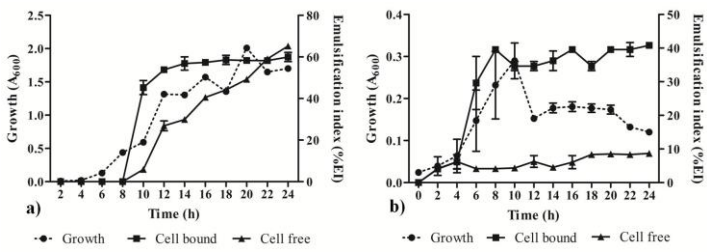

Figure 6 Time course of bioemulsifier production in ZM and AM1 media

As shown in figure. 6a , the cell-bound emulsification of *S. silvestris* AM1 starts to increase after 8th hour in AM1 medium as compared to ZM medium. But the Cell-free amyloid BE-AM1 release also starts simultaneously with cell-bound. Thus, in presence of production enhancing medium components, the cell-bound amyloid BE-AM1 expression is delayed by 4h, but, cell-free amyloid BE-AM1 release is preponed by 6h. The correlation of growth in AM1 and in ZM media showed There was higher correlation of growth of *S. silvestris* AM1 in ZM and AM1 media with r^2=0.895. **Fowler et al., (2006**) reported the carefully orchestrated amyloidogenesis and secretion pathway in animal tissues which can be used to study both amyloid pathology and melanin formation.

In comparison to growth in ZM medium or AM1 medium, the growth of *S. silvestris* AM1 was significantly low ($p< 0.0001$) in oligotrophic conditions. After 12th h of growth, the growth dropped drastically. Highest cell bound %EI observed in oligotrophic conditions was 41% as compared to 62.5% and 68% in ZM and AM1 medium. The cell free emulsification was least in comparison with AM1 and ZM medium even after 24h. The cell free emulsification shown is at the basal level, but enough for the emulsification activity to be visible (that is above the CMC level).

The ability of food proteins for fibrillation has been progressively recognized as an appealing strategy for broadening and improvement of food protein functionality as seen in case of heat induced amyloid- albumin protein (**Cao and Mezzenga, 2019; Pearce et al., 2007**). Pérez-**Tavarez et al., (2020)** reported the assembly of animal food allergens into amyloids in gastric-like environments and further interaction with IgE. Due to current understanding of the interaction of non-identical amyloids leading to pathological amyloidogenesis, this can prove to be interesting phenomenon to persue for further research. **Cao and Mezzenga, (2019**) studied the uses of food protein amyloids as Thickening and gelling agents, surface active agents, nutrient-drug carriers, packaging materials, antioxidents and as antimicrobial agents.

Evidences are accumulating for the cross-interactions of bacterial functional amyloids with pathological amyloids like α-synnuclein enhancing neurodegenerative diseases. While humans code almost 30 proteins with amyloidogenic properties, their cross-interaction with extensively studied functional amyloids from gut-associated bacteria can trigger amyloid accumulation and neurodegeneration (**Sampson et al., 2020**).

CONCLUSION

Recently, bacterial functional amyloids from gut microbiota have been implicated inn enhancement of pathological amyloid progression of neurodegenrative diseases. Hence, studies on bacterial functional amyloid production and the fators thereby influencing this amyloidogenesis has increased in importance. In present work, *S. silvestris* AM1 did not utilize major carbon sources for its BE-AM1 production, but could use basic protein sources like BSA and peptone for significant amyloid production. The unique ability of *S. silvestris* AM1 and factors influencing its amyloid bioemulsifier production can be further used biotechnologically for studying other strains for various biomolecule production of industrial potential.

Acknowledgments: AM is grateful to University Grants Commission (UGC), India for the financial grant.

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Supplementary table.

Supplementary table 1: Plackett – Burman design for 16 medium components of ZM

Trials	Variables																		
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X 10	X 11	X 12	X 13	X 14	X 15	X 16	D1	D2	D3
1	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-
2	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+
3	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+
4	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-
5	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-
6	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-
7	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-
8	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+
9	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-
10	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+
11	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-
12	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+
13	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+
15	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+
16	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-
17	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-
18	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+
19	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-