

Medium optimization for surfactant and chelator stable alpha amylase from Halotolerant bacterium

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Abstract

Little Rann of Kutch, a desert of Gujarat, India was selected to get an extremotolerant bacterium, and one potential isolate, capable to tolerate NaCl stress from upto 25% (w/v) was named Lrk-A. The isolate Lrk-A was screened for the extracellular alpha amylase production and starch agar plate showed a zone of starch utilization. Plackett-Burman design was used to identify and to optimize the nutrient components. The enzyme amylase is one of the hydrolysing enzymes used in detergent formulation and the effectiveness of the enzyme depends on its compatibility with other ingredients of the detergent formulation. In this study anionic surfactants Sodium Dodecyl hydrogen Sulphate (SDS), the cationic surfactant Cetyl Trimethyl Ammonium Bromide (CTAB), chelating agent Ethelene Diamine Tetra Acetic acid (EDTA) and reducing agent Urea were used to check the tolerance capacity of bacterial amylases from Lrk-A isolate. These compounds were checked at 10mM concentration with 24 hrs incubation period time. We found, SDS and CTAB have activating effect on amylase. This work reports that, this bacterial amylase from Lrk-A isolate have potential application in detergent industry.

Keywords: Alpha amylase, chelator, surfactant, halotolerant, Plackett-Burman design

Introduction

Among various extracellular enzymes, α -amylase ranks first in terms of commercial exploitation. Spectrum of applications of alpha amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, textile, paper and detergent industry. Amylases are a group of enzymes that have been found in several microorganisms like bacteria and fungi. The major advantage of using microorganisms for the production of amylase is the

economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. The microbial α -amylase for industrial purposes are derived mainly from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Aspergillus oryzae*. For any amylase to be commercialized it should be produced cheaply and should be potent for the industry application, in other words extremotolerant. The strain's production potential not only depends on the genetic nature but also on nutrient supply and cultural conditions. So it is important to

know the suitable nutrients and cultural conditions required to achieve higher productivity (Reddy et al., 2011).

Medium Optimization

Various methods for amylase production are used since long. One of the methods is solid state fermentation (SSF) which is popular due to its inherent advantages, eg. higher yield, improved oxygen circulation, less energy requirement, minimum efforts in downstream processing (Singh et al., 2010). Another method used for amylase production is submerged fermentation with its benefit of speedy bulk production and it is very widely used for industrial production. It is very important to produce amylase at cost benefit. So it has been a common practice to optimize the cultural and production parameters through the statistical approach as was used for cold active amylase from *Streptomyces* by Cotarlet and Bahrim (2012). Designing an appropriate fermentation medium is of crucial importance because medium composition can significantly affect product yield (Gao et al., 2009). The traditional 'one – factor at a time' technique used for optimizing a multi variable system is not only time consuming but also often easily misses the alternative effects between components (Kumar and Satyanarayana, 2007). The optimization of fermentation conditions, particularly nutrients and physiological parameters are of primary importance in the development of any fermentation process owing to their impact on the economy and practicability of the process. Statistical experimental designs have been used for several decades and it can be adopted at various phases of an optimization strategy, such as for screening experiments or for looking for the optimal conditions for targeted response (Box and Behnken, 1960). The results analyzed by statistically planned experiment are better acknowledged than those carried out by the traditional one variable at a time (OVAT).

Some of the popular choices in applying statistical design to bioprocessing include the Plackett-Burman design (Fattah et al., 2012) and Response surface methodology with various designs (Fattah et al., 2008; Sersy, 2012).

Effect of chelator and detergents on amylase activity

Many substances alter the activity of an enzyme by combining it in a way that influences the binding of the substrate. These substances are 'effectors'. Effectors can either be inhibitors or activators. Of the microorganisms, many bacterial and fungal strains have been explored for the production of α -amylase, mostly owing to low cost in production. However, in certain applications like detergent and bakery industries the properties of α -amylase needed is quite challenging and therefore continuous efforts are being undertaken for obtaining α -amylases that are tolerant under extreme environmental conditions, especially salt, pH and temperature. Enzymes that are capable of surviving under these adverse conditions are termed either as extremophilic or extremotolerant. Several extremophilic enzymes have been isolated and well characterized. However these differ from the extremotolerant enzymes as they are functional only under extreme conditions where as the extremotolerant enzymes are functional even under mesophilic conditions (Anupama and Jayaraman, 2011). Several salt tolerant bacterial strains from saltern of Kumta, coastal Karnataka, India are isolated and have shown proteases that are tolerant to salt and alkaline pH conditions (Pooja and Jayaraman, 2009). In continuation to unravel the potent amylase for potential application as survival in harsh condition. Many research groups have isolated salt tolerant bacterial strains from saltern of Bhavnagar and Okha seacost and desert of Kutch of Gujarat state.

As each application of α -amylase requires unique properties, e.g. thermostable α -

amylases have extensive commercial applications in starch processing, brewing and sugar production, search for novel thermoalkaline strains is necessity (Hanli et al., 2013). Similarly, detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (Hmidet et al., 2009; Mitidieri et al., 2006). These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate, etc. to dextrans and other smaller oligosaccharides (Mukherjee et al., 2009; Olsen and Falholt, 1998). Removal of starch from surfaces is also important in providing a whiteness benefit since starch can be an attractant for many types of particulate soils. Amylases used in detergent industry are from *Bacillus* or *Aspergillus* as examples (Mitidieri et al., 2006).

Apart from surfactants, chelators are a common ingredient in most laundry detergents. They have a number of different functions such as reducing water hardness by sequestering Ca^{2+} and Mg^{2+} , assisting in keeping particulate soil in suspension and the removal of certain stains, thus complementing the action of the anionic surfactants. But the presence of both the chelators and metal dependent enzymes in a liquid detergent presents a challenge. So, enzyme's stability in presence of chelators is very much important and so many attempts are made to find stable enzyme (Lund et al., 2011). Typically loss of activity of the amylase originates from a number of undesirable interactions with the other components of the detergents (e.g. surfactant, pH controlling agents, chelators, colorants, perfume, etc.) with surfactant-

induced unfolding being one of the most common modes of enzyme inactivation (Stoner et al., 2006; Lalonde et al., 1995). Common chelators used in laundry detergents are of three types: (1) Citrate- a weak chelator but has an excellent environmental profile. (2) DTPA- (an EDTA analogue) is considered to have better environmental profile and (3) HEDP. DTPA and HEDP are strong chelators, usually added for more specific purposes. For instance, target metal ions incorporated in many of the highly colored stain structures such as blood and tea (porphyrins and tannins respectively). Wash performance profile of HEDP is different to some extent from that of DTPA and EDTA described above, but like the latter two it is a key component of helping to remove transition metal ions present e.g. in some beverage stains such as tea and red wine (Watson, 2006). An interesting twist is that HEDP like other phosphorus containing chelators are banned in the US, while DTPA is banned in Europe due to concerns with aquatic toxicity (Watson, 2006). And so EDTA can be a better choice. As EDTA is not phosphorus containing chelator it has no environmental issue.

In this context the present study deals with optimizing nutritional conditions by using Plackett – Burman experimental design to maximize amylase production by desert bacterial isolate. Moreover the study was extended to evaluate the potency of the produced amylase for detergent industry.

Materials and methods

Strain and screening

Bacterial species Lrk-A was isolated from soil sample of Little Rann of Kutch Gujarat, India. And was screened for extracellular amylase production using starch agar plate (1% soluble starch, 0.2% Yeast extract, 0.5% Peptone, 0.05% MgSO_4 , 0.05% NaCl , 0.15% CaCl_2 , Agar 2%, pH-7). Zone of utilization was confirmed by using iodine

reagent (Iodine 1%, KI 3% prepared in Distilled water).

Salt tolerance

Salt tolerance range was checked by growing Lrk-A on Nutrient Agar medium containing from 0% to 25% (w/v) NaCl concentration.

Optimization of amylase production by submerged state fermentation:

Submerged fermentation was carried out in 250 ml Erlenmeyer Flask by taking 100 ml of starch broth medium prepared in potassium phosphate buffer 0.05M, pH-7 using ingredients and their levels as shown in Table: 1.

Table 1: Nutritional ingredients used in production medium.

No.	Ingredient %(w/v)	Low (-1)	High (+1)
1	Starch	0.2	0.8
2	YE	0.1	0.4
3	Peptone	0.2	0.8
4	BaCl ₂	0.04	0.16
5	MnCl ₂	0.04	0.16
6	NaCl	0.5	2.0
7	pH (dummy variable)	7	7

Medium was sterilized by autoclaving followed by cooling to room temperature and inoculated with 1% inoculum bacterial suspension. After inoculation, medium containing flasks were incubated at 28°C for 48 hrs on NOVA orbital shaker at 120 rpm.

Extraction & Enzyme assay:

After successful incubation, the enzyme was extracted for alpha amylase activity assay. Fermentation broth was centrifuged at 10,000 rpm for 10 minutes and clear supernatant was used as a source of the enzyme (Shah et al., 2014). A reactive mixture contained 0.5 ml of 1% w/v starch in 0.05 M potassium phosphate buffer, pH-

7.0 and 0.5 ml culture supernatant as amylase. The mixture was incubated at 37°C for 30 mins. The reducing sugar released was measured using 3,5 -dinitrosalicylic acid (DNSA). Resulting reducing sugar was measured at 540 nm (Miller, 1959). One unit (U) was defined as the enzymatic activity needed for the release of 1µmol of glucose equivalents per unit volume per assay time of reaction.

Identification of significant nutrients and their effect by Plackett-Burman design:

To identify the significant nutrients, Plackett-Burman design was used. Six variables were selected for this study keeping pH as dummy variable. Each variable was studied at two levels, 8 trials were carried out. Table-2 shows the Plackett-Burman experimental design with seven variables including level of each variable.

Statistical analysis

The statistical analysis of this data was performed using method given by Stanbury et al. (1997, pp. 110-112) to evaluate the Fishers f test for analysis of variance to determine the statistical significance of the model. A difference was considered statistically significant when $p < 0.05$.

Characterization of amylase for suitability to detergent industry:

Amylase enzyme was treated with SDS, CTAB, EDTA and Urea prepared in 0.05M Potassium Phosphate buffer, pH-7 at 10mM final concentration and was incubated at 37°C for 24 hrs. After 24 hrs incubation, amylase assay was performed as above mentioned method.

Results

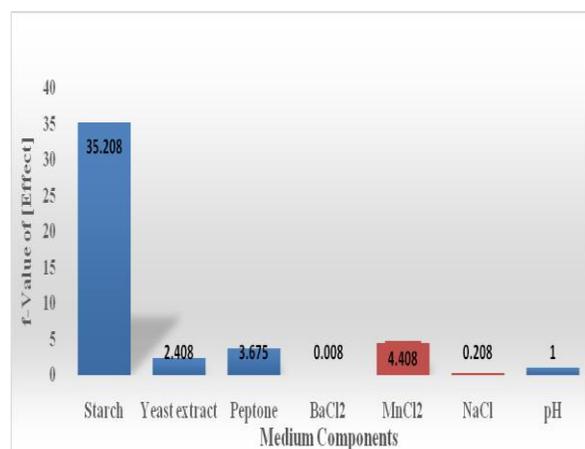
When probability tables are examined, it is found that factors Starch, MnCl₂, Peptone, Yeast Extract, shows large effect, which are very significant.

Table 2: Plackett-Burman experimental design for screening significant process variables affecting alpha-amylase.

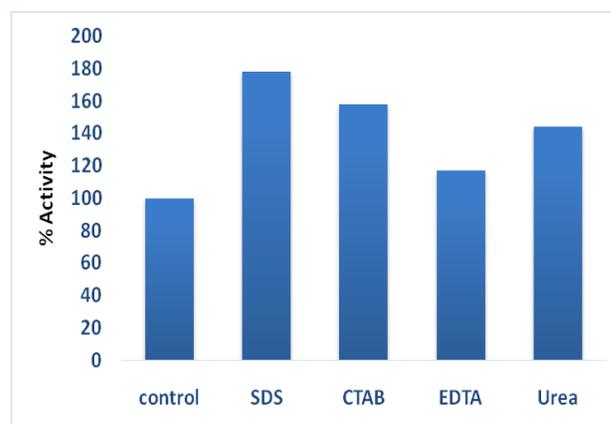
Trial	Starch	YE	Peptone	BaCl ₂	MnCl ₂	NaCl	pH	Yield (U)
1	+1	+1	+1	-1	+1	-1	-1	7.6
2	-1	+1	+1	+1	-1	+1	-1	5.2
3	-1	-1	+1	+1	+1	-1	+1	4.0
4	+1	-1	-1	+1	+1	+1	-1	5.4
5	-1	+1	-1	-1	+1	+1	+1	3.6
6	+1	-1	+1	-1	-1	+1	+1	8.2
7	+1	+1	-1	+1	-1	-1	+1	8.2
8	-1	-1	-1	-1	-1	-1	-1	3.6

Table 3: Analysis of the yields for culture Lrk- A.

	Starch	Yeast extract	Peptone	BaCl ₂	MnCl ₂	NaCl	pH
	A	B	C	D	E	F	G
∑(H)	29.4	24.6	25	22.8	20.6	22.4	24
∑(L)	16.4	21.2	20.8	23	25.5	23.4	21.8
Difference	13	3.4	4.2	-0.2	-4.6	-1	2.2
Effect	3.25	0.85	1.05	-0.05	-1.15	-0.25	0.55
Mean square	21.125	1.445	2.205	0.005	2.645	0.125	0.605
F-test (F _t =2.3)	35.208	2.408	3.675	0.008	4.408	0.208	1.008
Mean square for 'error' (dummy variable) = 0.6							

**Fig. 1: Pareto chart of nutrients and condition for the selection of significant variables for alpha amylase produced from Lrk-A (Orange colour shows negative effect and Blue colour shows positive effect of the nutrient component).**

Effect of Chelator and Surfactants on Enzyme stability

**Fig. 2: Amylase stability checked for 24 hrs at 10mM of surfactants, chelator and urea, considering control activity as 100%.**

Alpha amylase from Lrk-A is stable even after 24 hr treatment period with SDS CTAB, EDTA and Urea. The interesting fact is, amylase activity is enhanced in presence of 10mM SDS by 80%, by CTAB 60%, by EDTA 20%.

Discussion

Table-2 represents the experimental design and results obtained for α -amylase production. The analysis of variance (ANOVA) performed for these significant variables. For Plackett-Burman design as shown in (Table-3), the variables with p-value less than 0.05 (<0.05) were considered as significant.

The responses taken from Table-3 reveal that all the four significant variables have remarkable effects on α -amylase production. Starch as carbon source for α -amylase medium showed the highest level of significance. As described by Gangadharan et al. (2006), Starch can be a preferable carbon source over glucose due to feedback inhibition caused by reducing sugars for α -amylase production in *Bacillus* sp. These results confirmed the suggestion of Tanyildzi et al. (2006) that concentration of starch has a direct effect on α -amylase production. Organic nitrogen sources produce high α -amylase yield as compared to inorganic nitrogen sources from several *Bacillus* sp. (Aiyer, 2004; Swain et al., 2006; Sumrin et al., 2011). In our study, we observed significant effect of yeast extract and peptone on α -amylase production. High F-value (35.02) and significant p-value (0.05) of starch revealed that it could act as limiting variable as yeast extract and peptone in α -amylase production. A small variation in starch and peptone and yeast extract concentration can alter α -amylase yield as cleared from observed values in Table-3.

Negative effect of $MnCl_2$ suggest, decreasing the $MnCl_2$ level will enhance amylase production. Absence of positive effect of NaCl shows that although bacterial isolate Lrk-A is extreme halotolerant, it is not dependent on higher need or requirement of NaCl for amylase production. The Pareto chart of nutrients used (Figure-1) are the graphical representation to visualize the effect of component (Sumrin et al., 2011).

Effect of co-factor and other additives on the enzyme activity:

The enhancing effect of negatively charged surfactant, positively charged surfactant and chelator on amylase activity is very much important characteristic of the enzyme studied. This is in contrast to some reports as in *Haloarcula hispanicae* (Hutcheon et al., 2005), *Bacillus* sp.I-3 (Goyal et al., 2005), in *Mucor* sp. (Mohapatra, 1998), in *Bacillus aquimaris* VITPG (Anupama and Jayaraman, 2011) there is inhibiting effect. That means the enzymes are metalloenzymes. This suggest for metal independent amylase there will not be adverse effect of EDTA chelating agent but suggest stability of the enzyme to EDTA (Prakash et al., 2011).

Apart from this as is known from literature, most of the α -amylases calcium dependent. And formulation of enzyme-containing liquid detergents is a significant challenge due to the destabilizing interaction between several detergent components and the enzymes. As the addition of chelators may thus be expected to reduce their stability by chelating Ca^{2+} of the enzyme. Here we have shown that EDTA did not cause significant destabilization of amylase, That may be because of partial independence from Ca^{2+} . As Ca^{2+} was not medium component not the assay component.

These results have important implications for enzyme containing detergent formulations.

Conclusion

In the present study, a novel α -amylase from halotolerant Lrk-A isolate from Kutch desert was checked for its stability at surfactants, chelator and reducing agent Urea. And results, clearly indicate that as the enzyme is stable with surfactants and chelator simultaneously and its partial independence from Ca^{2+} make it a good candidate for application in laundry detergents and stability at urea, make it suitable for various application in starch industries.

For Further work with this extremotolerant amylase medium optimization at fine level with Response surface methodology is suggested and characterization of purified amylase for potential use is suggested.

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Conflict of interest: None

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