# Effect of amino acids on the repression of alkaline protease synthesis in haloalkaliphilic *Nocardiopsis dassonvillei*

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A B S T R A C T

A newly isolated salt-tolerant alkaliphilic actinomycete, *Nocardiopsis dassonvillei* strain OK-18 grows on mineral salts medium with glucose as carbon source. It also grows and produces protease with amino acids as sole carbon source. The synthesis of extracellular alkaline protease parallel to growth was repressible by substrate concentrations. The absolute production of the protease was delinked with growth under nutritional stress, as protease production was high, despite poor growth. When amino acids served as the sole source of carbon and nitrogen, the enzyme production was significantly controlled by the number of amino acids. Maximal protease production was achieved with proline, asparagine, tyrosine, alanine, methionine and valine as sole source of carbon and nitrogen in minimal medium. With the increasing number of different amino acids in the presence and absence of glucose, the protease production was synergistically lower as compared to complex medium.

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## 1. Introduction

Studies on the marine actinobacteria in recent years have emerged as one of the major aspects of research [1]. Actinobacteria have gained significance not only due to their taxonomy, diversity and ecological significance, but also on account of their enzymes, unique metabolites and bioactive compounds [1,2].

Proteases constitute one of the most important groups of industrial enzymes and ubiquitously present in all organisms. They have potential applications in different industries that include laundry detergent, food, pharmaceutical, peptide synthesis, leather, meat processing, silk and silver recovery from used X-ray films [3,4]. The catalysis and stability of the microbial proteases under wide range of conditions including high salinity and alkaline pH is one of the major requirement and challenge. In this context, the enzymes from the haloalkaliphilic organisms that can function at low and high concentrations of NaCl over a broad range of pH appear promising [5].

Microbial alkaline proteases are widely reported from *Bacilli* and *Streptomyces* [6,7]. However, protease production and characterization from actinomycetes, particularly *Nocardiopsis*

has not achieved similar attention. Most of the work on actinomycetes relate to antibiotics and other bioactive compounds [8].

Proteases production in microorganisms is generally constitutive or partially inducible. However, only limited knowledge exists on the mechanisms which regulate the protease synthesis and its secretion [9–12]. The production of extracellular serine protease [13] in microorganisms is strongly influenced by the environmental parameters and media components, for instance, variations in C/N ratio, presence/absence of metabolizable sugars, such as glucose [14] and rapidly metabolizable nitrogen sources, such as amino acids. Besides, several other factors, such as aeration, inoculum load, medium pH, temperature and incubation time [15,16] and metal ions [17] play important role in protease synthesis.

Secretion of the alkaline proteases from actinomycetes is dependent on the growth rate and availability of the carbon and nitrogen sources in the medium [18]. Various nitrogen sources including amino acids at certain concentrations repress the enzyme production. Repression of the synthesis of biosynthetic enzymes by the end product of their action is an important aspect of the metabolic regulation in microorganisms. [19]. Enzyme repression is a mode of regulation through which the synthesis of an enzyme is prevented by repressor molecules. As a result, end product acts as a feed-back-co repressor in association with intracellular apo-repressor and blocks the function of an operator [20].

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The production of an enzyme demonstrates a relationship with the growth of the organism. Generally, the protease synthesis and secretion are induced by peptides or other proteinaceous substrates. Amino acids repress protease synthesis even at low concentrations [21]. However, production of protease is controlled by numerous complex mechanisms during the transition of the exponential and the stationary phase [22,23]. Various nitrogen sources are known to repress enzyme production in Gram negative bacteria and Actinobacteria [24,25]. However, similar studies on the repression of the enzymes in haloalkaliphilic actinomycete, *Nocardiopsis dassonvillei* have not been earlier reported.

Many studies on the actinomycetes from the temperate regions have been carried out [1]. Till now, terrestrial soils and sediments are the predominant and widely explored sources for the actinomycetes. However, studies on the salt tolerant haloalkaliphilic actinobacteria are quite rare. Haloalkaliphilic actinomycetes and their proteases from the saline habitats of the coastal Gujarat have been studied during the last several years [2,10,11,26–29]. In the present report, we describe the effect of amino acids on the production of alkaline protease in a newly isolated marine actinomycete, *Nocardiopsis dassonvillei* OK-18 from the Okha Port (22.4667°N 69.0833°E), Arabian Sea.

## 2. Material and methods

### 2.1. Chemicals

Casein was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Gelatin, pure amino acids (L form), Minimal Davis Broth (Ingredients g/L: Dipotassium phosphate, 7.0 g; Monopotassium phosphate, 2.0 g; Sodium citrate, 0.5 g; Magnesium sulphate, 0.1 g; Ammonium sulphate,1.0 g) and other media components were purchased from Hi-Media Laboratories (Mumbai, India). All other chemicals used were of highest purity grade.

### 2.2. Microorganism and culture conditions

The extracellular alkaline protease producing haloalkaliphilic actinomycete was isolated from sea water near Okha Port (22.4667°N 69.0833°E), Gujarat (India). Sea water samples without dilution were subjected to thermal treatment by heating in a water bath at 60–70 °C for 30–60 min to reduce the number of undeniable bacteria. Actinomycetes were isolated at 30 °C using selective media. Further identification was based on the 16S rRNA gene sequencing of the isolate.

### 2.3. Enzyme repression by various amino acids

The repressive effect of amino acids was studied as described earlier [26], with some modifications in minimal medium with 5% (w/v) NaCl and 0.5% (w/v) glucose as the sole source of carbon without any other nitrogen source at pH 11.0. The amino acids were selected on the basis of charge, side chain and polarity. The stock solutions of the amino acids were filter-sterilized and added to a final concentration of 0–1% (w/v) in the sterilized minimal medium. Fifty millilitres of the production medium in a 250 mL flask was inoculated with 3% of 48–72 h old grown seed culture (optical density of 1.0 at $540_{nm} \sim 10^7$ cells/mL) and incubated at 30 °C under shaking conditions (120 rev/min) for 264 h. The culture aliquots were collected and centrifuged to obtain cell-free supernatant, followed by the measurement of growth. The cell free culture filtrates were used as a crude enzyme preparation and ratio of enzyme production and growth was calculated.

### 2.4. Enzyme assay

Alkaline protease was estimated by Anson-Hagihara method [30], using casein as substrate. One unit of the alkaline protease activity (U) was described as the enzyme liberating 1 μg of tyrosine per min under the assay conditions. The estimations were based on a tyrosine calibration curve.

### 2.5. Effect of the increasing number of amino acids on protease production

In order to investigate the influence of the combinations of the amino acids on protease production, OK-18 was grown in minimal media, pH 11.0, supplemented with 5%, (w/v) NaCl and increasing number of amino acids; in the presence and absence of 0.5% (w/v) glucose. The L-amino acids were added, each at the concentration of 1% (w/v), in the combinations; (1) proline, (2) proline, glutamine, (3) proline, glutamine, valine, (4) proline, glutamine, valine, isoleucine, (5) proline, glutamine, valine, isoleucine, glutamic acid, (6) proline, glutamine, valine, isoleucine, glutamic acid, threonine, (7) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, (8) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, serine, (9) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, serine, arginine hydrochloride, (10) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, serine, arginine hydrochloride & glycine. The medium was inoculated with 3% inoculums and after 264 h growth at 30 °C; cell mass and protease were monitored.

### 2.6. Effect of non polar side chain amino acids on protease production

Combinations of non-polar side chain amino acids were included in the medium as described above. The amino acids, each at the concentration of 1% (w/v), were added in combinations of glycine, alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine and tryptophan. The medium was inoculated with 3% inoculums and after 264 h growth at 30 °C; cell mass and protease were monitored as described above.

### 2.7. Effect of uncharged polar side chain amino acids on protease production

Effect of uncharged polar side chain amino acids on protease production in OK-18 was assessed in minimal mediathat contained 5%, (w/v), NaCl at pH 11.0 in the presence and absence of glucose, 0.5% (w/v). The amino acids, each at the concentration of 1% (w/v), were added in combination of serine, threonine, asparagine, glutamine, tyrosine and cysteine. The medium was inoculated at 3% of the inoculums and after 264 h growth at 30 °C; cell mass and protease were monitored.

### 2.8. Effect of essential and non essential amino acids on protease production

Effect of essential and non essential amino acids on protease production in OK-18 was assessed in minimal media that contained 5%, (w/v), NaCl at pH 11.0 in the presence and absence of glucose, 0.5% (w/v). The amino acids, each at the concentration of 1% (w/v), were added in combinations of histidine, isoleucine, methionine, phenylalanine, threonine, tryptophan and valine. And for non essential amino acids, each at the concentration of 1% (w/v), were added in combination of alanine, arginine, asparagine, aspartic acid, glutamine, glycine, proline, serine and tyrosine. The medium was inoculated at 3% of the inoculums and after 264 h growth at 30 °C; cell mass and protease were monitored.

## 2.9. Optimization of production medium for growth and protease secretion

### 2.9.1. Growth kinetics

The growth and alkaline protease production was studied at 30 °C in a medium containing (w/v) 1% gelatin, 5% NaCl; pH 11.0. The culture was incubated under shake flask conditions (120, rev/min) and at 24 h intervals, the growth was monitored at 540 nm.

## 2.10. Repression studies with carbon and nitrogen sources

The catabolite repression of the enzyme synthesis was assessed in Gelatin broth medium with carbon (glucose and fructose) and organic and inorganic nitrogen sources (yeast extract, malt extract and peptone) at 0–1.0%, (w/v) concentrations with optimum NaCl and pH. Growth and protease production were measured after 264 h of the incubation at 30 °C under shake flak conditions (120, rev/min).

## 2.11. Effect of NaCl on growth and protease production

The effect of salt on growth and protease production was studied in Gelatin Broth at NaCl concentrations of 0–15%, (w/v), with optimum pH. The growth and enzyme activity were quantified for 10–15 days at 24 h intervals.

## 2.12. Effect of pH on growth and protease production

The influence of pH on growth and protease production was studied in Gelatin Broth at pH 7–11 and 5% (w/v) NaCl concentration at 30 °C under shaking conditions at 120, rev/min. The growth and enzyme production was measured for 10–15 days at 24 h intervals.

## 2.13. Effect of Gelatin and different additives on the growth and protease production

The influence of gelatin and metal ions on growth and protease production was monitored in Gelatin broth that contained 0–5% (w/v) of gelatin at the optimum NaCl and pH under shake flask conditions (120, rev/min) at 30 °C for 10–15 days at 24 h intervals.

## 3. Results

In this study, we report the production of an alkaline protease by *Nocardiopsis dassonvillei* OK-18 under the influence of amino acids. Industrially important enzymes are expected to withstand extreme conditions of high temperature, broad range of pH and presence of inhibitors, besides having high catalytic activity and substrate specificity [2]. Therefore, now-a-days, there is an emerging interest in these enzymes [31].

## 3.1. Microorganism and culture conditions

The organism was Gram-positive with a filamentous structure and identified as *Nocardiopsis dassonvillei* on the basis of 16S rRNA gene sequencing. The isolate was also characterized for it's morphological, physiological and biochemical properties. The cell wall sugars and amino acids were also analysed as given in Bergey's Manual of Determinative Bacteriology. The 16S rRNA gene sequence was submitted to NCBI, as *Nocardiopsis dassonvillei* OK-18 (GenBank accession number KC119570). This is the first report of a haloalkaliphilic actinomycete, *Nocardiopsis dassonvillei* from the Arabian Seawater, at Okha Port Gujarat, India.

## 3.2. Enzyme repression by various amino acids

### 3.2.1. Repression of protease production by methionine, alanine, and leucine

Class 1-amino acids; methionine, leucine and alanine significantly supported the growth of OK-18. However, the protease production was relatively better in alanine (1.0%) even though growth was adversely affected (Fig. 1). Alanine, up to 1.0% (w/v) induced the protease production (148.32 ± 2.72 U/mL), while the growth was significantly favoured by leucine when compared with alanine and methionine in minimal medium. With alanine at 0.5% (w/v), the protease production was lower as compared to control. However, the protease production gradually decreased when both amino acids were added together in the minimal medium. At 0.5% (w/v) of methionine, alanine and leucine, the protease production was 52.85 ± 0.74, 17.59 ± 1.04 and 66.16 ± 1.16 U/mL, respectively. The protease production was significantly reduced with 1.0% (w/v) leucine, as compared to alanine and methionine.

Interestingly, while the cell growth decreased with the increasing concentrations of the amino acids from 0 to 0.5% (w/v), the synthesis of the alkaline protease and ratio of the enzyme activity versus growth increased with the Class-1 amino acids.
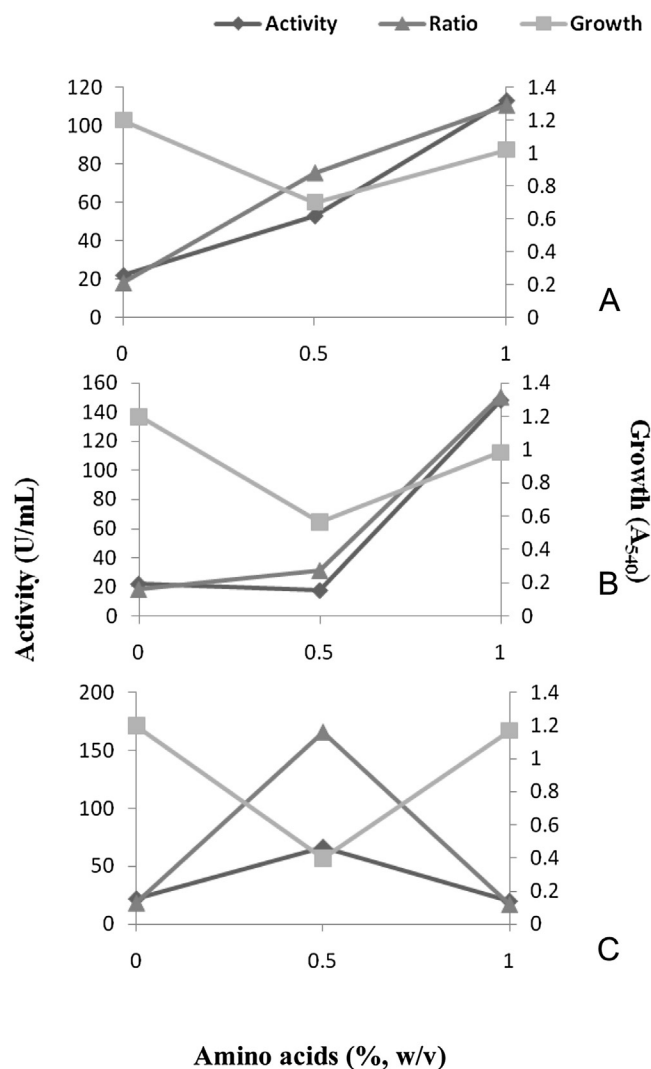


**Fig. 1.** Effect of Class-1 amino acids (0–1.0% w/v); methionine (A), alanine (B) and leucine (C) on the growth and protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions in presence of glucose in minimal medium.

Interestingly, as concentration increased from 0.5 to 1.0% (w/v), the cell growth increased in all three Class-1 amino acids. It suggested that the absolute production of the enzyme was delinked with growth. Further, with leucine, as the concentration of the amino acid increased from 0.5 to 1.0%, both, enzyme production and ratio decreased, suggesting an adverse effect of this amino acid on the synthesis of the alkaline protease. The repression of the protease synthesis by leucine was stronger as compared to other two amino acids of class 1. On increasing the concentration of leucine from 0.5 to 1.0 (%), the growth of the organism enhanced, while the protease production did not, which indicated that the absolute production of the enzyme was not linked with the growth.

### 3.3. Repression by aromatic amino acids

The finding with the Class-2, aromatic amino acids, confirmed maximum growth with phenylalanine as compared to tryptophan and tyrosine at 1.0% (w/v) concentration. The protease production was highest with 1.0% (w/v) tyrosine (143.06 ± 2.56 U/mL),

followed by 55.62 ± 1.87 U/mL in phenylalanine and 38.34 ± 1.73 U/mL in tryptophan (Fig. 2). While growth of the organism increased, the enzyme production significantly increased when phenylalanine was enhanced from 0.5 to 1.0% (w/v). Interestingly, with tryptophan and tyrosine, when concentration increased from 0.5 to 1.0% (w/v), the growth decreased while enzyme production and ratio increased.

At 1% (w/v), tyrosine induced the synthesis of the alkaline protease in minimal medium, while at the same concentration, phenylalanine and tryptophan had strong repressive effect when compared with tyrosine. All the three amino acids displayed induction of the enzyme at 1.0% (w/v) concentration when compared with control.

The trends clearly suggest the impact of aromatic amino acids on the synthesis of the protease in actinobacteria. Interestingly, with the increasing concentrations of tyrosine, the growth of the organism decreased, while the synthesis of alkaline protease and ratio of the enzyme production versus growth increased. It clearly indicated the induction of the synthesis of alkaline protease.

### 3.4. Repression by aspartic acid

Aspartic acid, a representative of class 3 amino acids, has polar uncharged R group. It significantly supported the growth and protease production. Maximum protease production (61.4 ± 1.2 U/mL) was observed with 0.5% (w/v) aspartic acid, while at 1.0%, it was reduced to 40.1 ± 1.25 U/mL, which was observed higher than the control (Fig. 3). With the increasing concentration of asparatic acid from 0.5 to 1.0% (w/v), the growth increased, while there was decrease in the enzyme production and the ration of the enzyme production and growth. The findings suggested that aspartic acid plays significant role in the synthesis of alkaline protease in minimal medium.

### 3.5. Repression by histidine and arginine

Histidine and arginine with charged R-groups represent class 4 amino acids. Both, histidine and arginine, at 0.5% (w/v), significantly repressed the enzyme production when compared with control (0%; w/v). Maximum enzyme production (18.51 ± 1.68 U/mL) was observed in 0.5% (w/v) arginine, while at 1.0% (w/v), the amino acid significantly repressed the enzyme production to a low level of 10.8 ± 1.95 U/mL (Fig. 4). Arginine at 1.0% (w/v) favourably supported the growth as compared to histidine. Over all, with respect to the Class-4 amino acids, as the concentration increased from 0.5 to 1.0 (%), the growth considerably increased, while the enzyme production decreased.
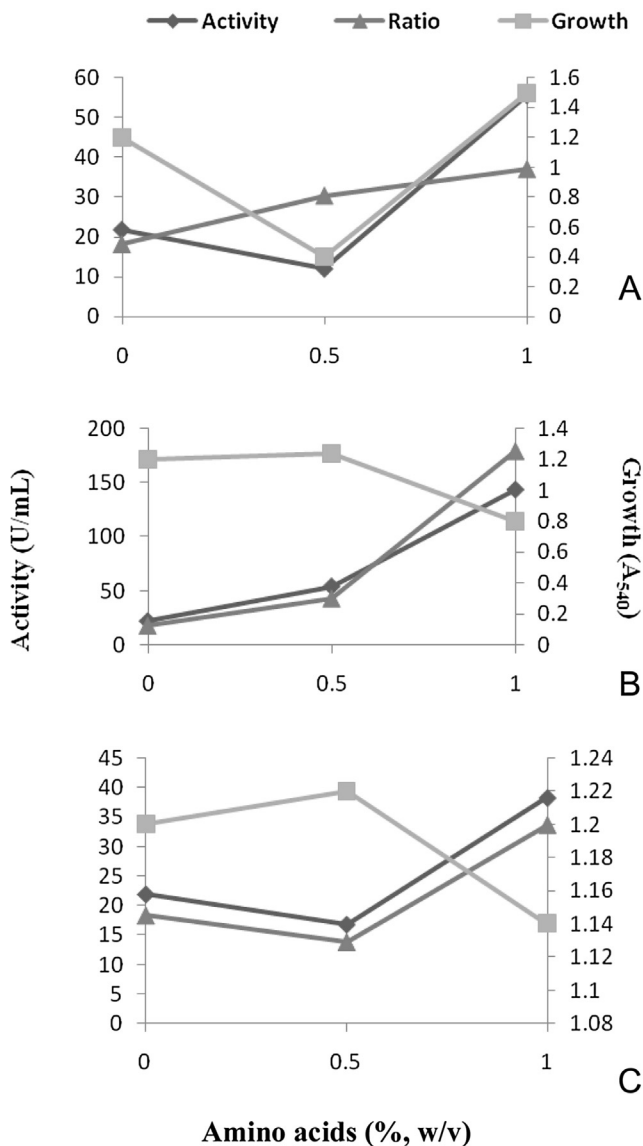
**Fig. 2.** Effect of Class-2 amino acids (0–1.0% w/v); phenylalanine (A), tyrosine (B) and tryptophan (C)on growth and protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions in presence of glucose in minimal medium.

**Fig. 3.** Effect of class-3 amino acid (0–1.0% w/v); aspartic acid on growth and protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions in presence of glucose in minimal medium.
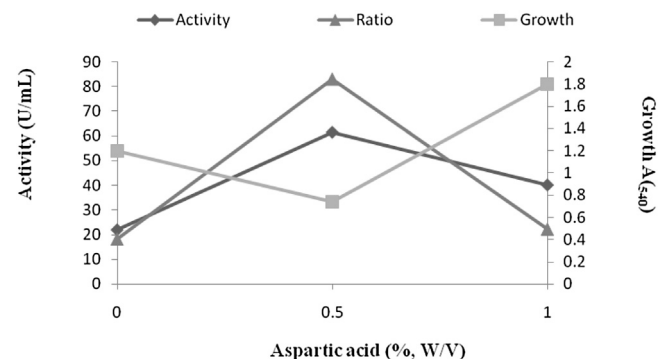
**Fig. 4.** Effect of class-4 amino acids (0–1.0% w/v); histidine (A), arginine (B) on growth and protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions in presence of glucose in minimal medium.
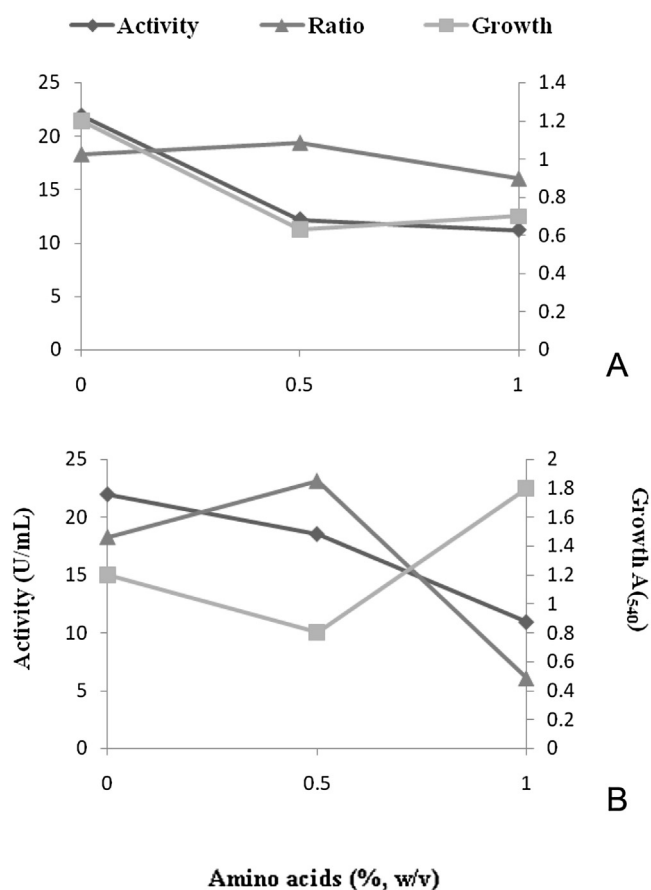
### 3.6. Repression by asparagine

Aspargine, a representative of Class-5 amino acids with charge R group, favoured growth of the organisms, while the enzyme production was repressed to the level of $13.81 \pm 1.55$ U/mL in 0.5% (w/v) amino acid. At 1.0% (w/v), asparagine, however, enhanced the enzyme production to $105.59 \pm 2.32$ U/mL. The growth and protease production increased with increasing concentrations of asparagine from 0.5 to 1.0% (w/v) (Fig. 5). The trend indicated that
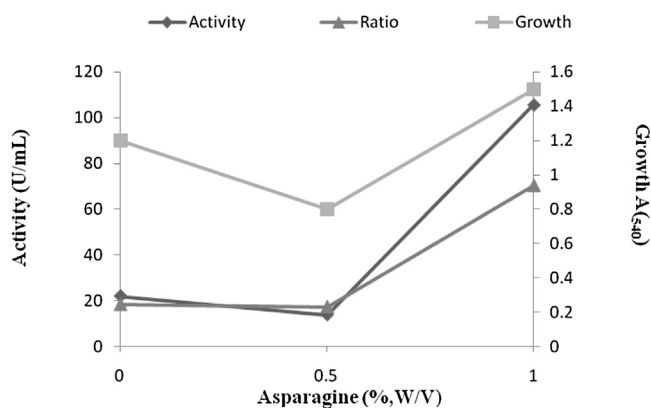


**Fig. 5.** Effect of class-5 amino acid (0–1.0% w/v); asparagine on growth and protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions in presence of glucose in minimal medium.

with increasing concentrations of the amino acid up to 0.5% (w/v), the growth and enzyme production decreased as compared to control followed by enhancement at concentrations above 0.5% (w/v).

### 3.7. Repression of protease production by other amino acids

The repression of the alkaline protease by different amino acids is documented in Table 1. The enzyme production was $171.19 \pm 1.85$ U/mL, $81.92 \pm 1.69$ U/mL, $93.71 \pm 3.51$ U/mL and $37.17 \pm 2.05$ U/mL with proline, glycine, valine and serine, respectively, at 1.0% (w/v). In the presence of 1.0% (w/v) glycine and valine, the enzyme production was highly reduced. When threonine and isoleucine were added into the medium along with glucose, the cell density was enhanced as compared to control devoid of amino acids. Addition of 1.0% (w/v) proline into the basal medium with glucose enhanced the cell density, while valine and glycine at 0.5 and 1.0%, respectively, inhibited the growth of the organism.

### 3.8. The additive effect of amino acids on the protease production

Increasing number of different amino acids in growth medium markedly enhanced the growth up to a combination of five amino acids. However, when number of amino acids increased to more than five, there was an enhancement in the growth, while the enzyme production decreased. Maximum protease production was evident with the combination of proline + glutamine + valine + isoleucine + glutamic acid in the presence of glucose (Fig. 6). Maximum protease production was observed with six different amino acids, wherein amino acids acted as the sole source of carbon and nitrogen while glucose was omitted from the medium.

### 3.9. Effect of increasing number of the non- polar side chain amino acids on growth and protease production

In this study effect of non-polar side chain amino acids on growth and protease production was assessed in various combinations with and without glucose. At 1.0% (w/v) amino acids with glucose $24.46 \pm 2.84$ U/mL activity was observed. The non polar side chain amino acids promoted growth in minimal medium in the presence of glucose, while the protease synthesis was inhibited. In the absence of glucose, the non-polar amino acids in the minimal medium supported the protease production with enhanced protease synthesis (Fig. 6). Interestingly, when the same experiment was carried out in the absence of glucose, the growth was marginally enhanced (Fig. 6), which implies that under repressed conditions amino acids served as the sole source of carbon.

### 3.10. Effect of increasing number of the uncharged polar side chain amino acids on growth and protease production

In the presence of glucose, in combination of the uncharged polar side chain amino acids (serine, threonine, asparagine, glutamine, tyrosine and cysteine) at 1.0% (w/v) each in the minimal medium, the enzyme activity was $16.40 \pm 2.9$ U/mL, while in control, without any amino acids, the enzyme activity was 21.94 U/mL. The results suggested that the uncharged polar side chain amino acids with glucose supported growth of the organism rather than the production of the protease (Fig. 6). In the absence of glucose, while the cell density was low, the enzyme production reached to $61.25 \pm 3.55$ U/mL, indicating a fourfold enhancement.

**Table 1**
Effect of amino acids on the protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions in presence of glucose in minimal medium. All the data are the mean ± SD, n = 3; SEM – standard error of mean.

| Amino acid | Enzyme yield U/mL | | | | Yield Index (fold) | |
|---|---|---|---|---|---|---|
| | 0.5% | | 1.0% | | 0.5% | 1.0% |
| | | SEM | | SEM | | |
| Control | 21.94 ± 1.01 | 0.58 | 21.94 ± 1.01 | 0.58 | 1.00 | 1.00 |
| ʟ-Glutamine | 38.01 ± 1.83 | 1.05 | 24.1 ± 0.9 | 0.51 | 1.73 | 1.09 |
| ʟ-Serine | 25.53 ± 2.71 | 1.56 | 37.17 ± 2.05 | 1.18 | 1.16 | 1.69 |
| ʟ-Cystine | 17.26 ± 1.24 | 0.71 | 8.66 ± 0.68 | 0.39 | 0.78 | 0.39 |
| ʟ-Valine | 19.38 ± 2.42 | 1.40 | 93.71 ± 3.51 | 2.02 | 0.88 | 4.27 |
| ʟ-Proline | 114.16 ± 1.66 | 0.95 | 171.19 ± 1.85 | 1.06 | 5.20 | 7.80 |
| ʟ-Isoleucine | 14.18 ± 1.59 | 0.92 | 11.18 ± 1.18 | 0.68 | 0.64 | 0.50 |
| ʟ-Glycine | 14.85 ± 1.35 | 0.77 | 81.92 ± 1.69 | 0.97 | 0.67 | 3.73 |
| ʟ-Glutamic acid | 35.51 ± 1.51 | 0.87 | 21.81 ± 1.19 | 0.68 | 1.61 | 0.99 |
| ʟ-Histdine hydrochloride | 12.21 ± 0.215 | 0.12 | 11.2 ± 1.2 | 0.69 | 0.55 | 0.51 |
| ʟ-Threonine | 22.26 ± 1.24 | 0.71 | 17.01 ± 2.01 | 1.16 | 1.01 | 0.77 |
| ʟ-Phenylalanine | 12.17 ± 0.42 | 0.24 | 55.62 ± 1.87 | 1.08 | 0.55 | 2.53 |
| ʟ-Asparagine | 13.81 ± 1.55 | 0.89 | 105.58 ± 2.32 | 1.34 | 0.62 | 4.81 |
| ʟ-Tyrosine | 53.83 ± 1.83 | 1.05 | 143.06 ± 2.56 | 1.47 | 2.45 | 6.52 |
| ʟ-Alanine | 17.59 ± 1.04 | 0.60 | 148.32 ± 2.72 | 1.57 | 0.80 | 6.76 |
| ʟ-Tryptophan | 16.48 ± 2.06 | 1.19 | 38.34 ± 1.73 | 1.0 | 0.75 | 1.74 |
| ʟ-Leucine | 66.16 ± 1.16 | 0.66 | 19.88 ± 2.68 | 1.54 | 3.01 | 0.90 |
| ʟ-Aspratic acid | 61.4 ± 1.2 | 0.69 | 40.1 ± 1.25 | 0.72 | 2.79 | 1.82 |
| ʟ-Arginine hydrochloride | 18.5 ± 1.68 | 0.97 | 10.9 ± 1.95 | 1.12 | 0.84 | 0.49 |
| ʟ-Methionine | 52.85 ± 0.74 | 0.43 | 112.8 ± 1.90 | 1.10 | 2.40 | 5.14 |

### 3.11. Effect of essential and non essential amino acids on protease production

The combination of essential and non-essential amino acids on the protease production was assessed in minimal medium in the presence and absence of glucose. At 1.0% (w/v) concentration of the amino acids with glucose, the enzyme production was 44.65 ± 3.21 U/mL and 54.50 ± 2.5 U/mL with essential and non-essential amino acids, respectively (Fig. 6). The essential amino acids as compared to non-essential amino acids along with glucose in minimal medium promoted growth.

The protease production was 68.15 ± 1.15 U/mL and 107.85 ± 4.15 U/mL with essential and non-essential amino acids, respectively, in the absence of glucose. Overall in the absence of glucose, the cell density was quite low. However, the non-essential amino acids in minimal medium without glucose supported better growth as compared to the essential amino acids. On the whole, the trend suggests that the non-essential amino acids favoured the synthesis of alkaline protease in the medium.

### 3.12. Optimization of the production medium for growth and protease production

#### 3.12.1. Growth characteristics

OK-18 grew in the range of 28–30 °C, optimally at 30 °C, in Gelatin broth. The organism grew well at pH 8–11 and NaCl concentrations of 0–15% (5% optimum), which indicated halo-alkaliphilic nature of the strain. The growth kinetics with reference to protease production suggested that the protease production started after 144 h and reached to an optimum level at 264 h (Supplement Fig. 1). A prolonged incubation beyond this period did not further add to the enzyme yield. Supplement Fig. 2 represents the gel picture for SDS-PAGE analysis of the crude alkaline protease.

### 3.13. Effect of inoculum load and agitation rate

Production of the extracellular enzyme and the growth of the organism are affected by the rate of inoculum and its size [32].

Different sizes of the inoculums, 1–15% (v/v), were added into the medium to study the protease production. The maximum enzyme activity was evident at an inoculum load of 3% (v/v), with a decline in the enzyme production at 10% (v/v) inoculum. With the increasing size of the inoculum, the production of the enzyme declined probably due to the exhaustion of the nutrients in the fermentation medium.

### 3.14. Repression studies with carbon and nitrogen sources

The production optimization of the alkaline protease in submerged fermentation demonstrated the impact of various carbon and nitrogen sources on the gross yield of the enzyme and repression of its synthesis in actinobacteria. The effect of different carbon and nitrogen sources on protease production is documented in Supplement Table 1. The results revealed that in the presence of carbohydrate, the growth was increased; however, it negatively affected the protease production. Most of the carbon sources in the medium demonstrated catabolic repression. Glucose repressed the enzyme synthesis as compared to the control, when glucose was absent. However, the presence of glucose promoted the growth of the organisms. With 0.5% (w/v) glucose, the enzyme production was 41.36 ± 3.46 U/mL, which gradually decreased to 21.8 ± 1.74 U/mL when glucose concentrations increased from 0.5 to 1.0% (w/v). Among the various carbon sources, fructose was found to enhance both growth and protease production. Interestingly, with the increasing concentrations of fructose, the enzyme production also increased.

With respect to the nitrogen sources, such as peptone, yeast extract and malt extract, the enzyme production gradually decreased with increasing concentrations of the nitrogen sources, as revealed in Supplement Table 1. Taken together, the inorganic and organic nitrogen sources employed in this study, protease production was positively affected.

With increasing concentrations of the complex nitrogen sources, the enzyme production gradually decreased. With 0.5% yeast extract, peptone, malt extract and casein, the enzyme activities were 42.62 ± 1.06 U/mL, 162.8 ± 2.2 U/mL, 176.88 ± 3.42 and 66.51 ± 1.80 U/mL, respectively (Supplement
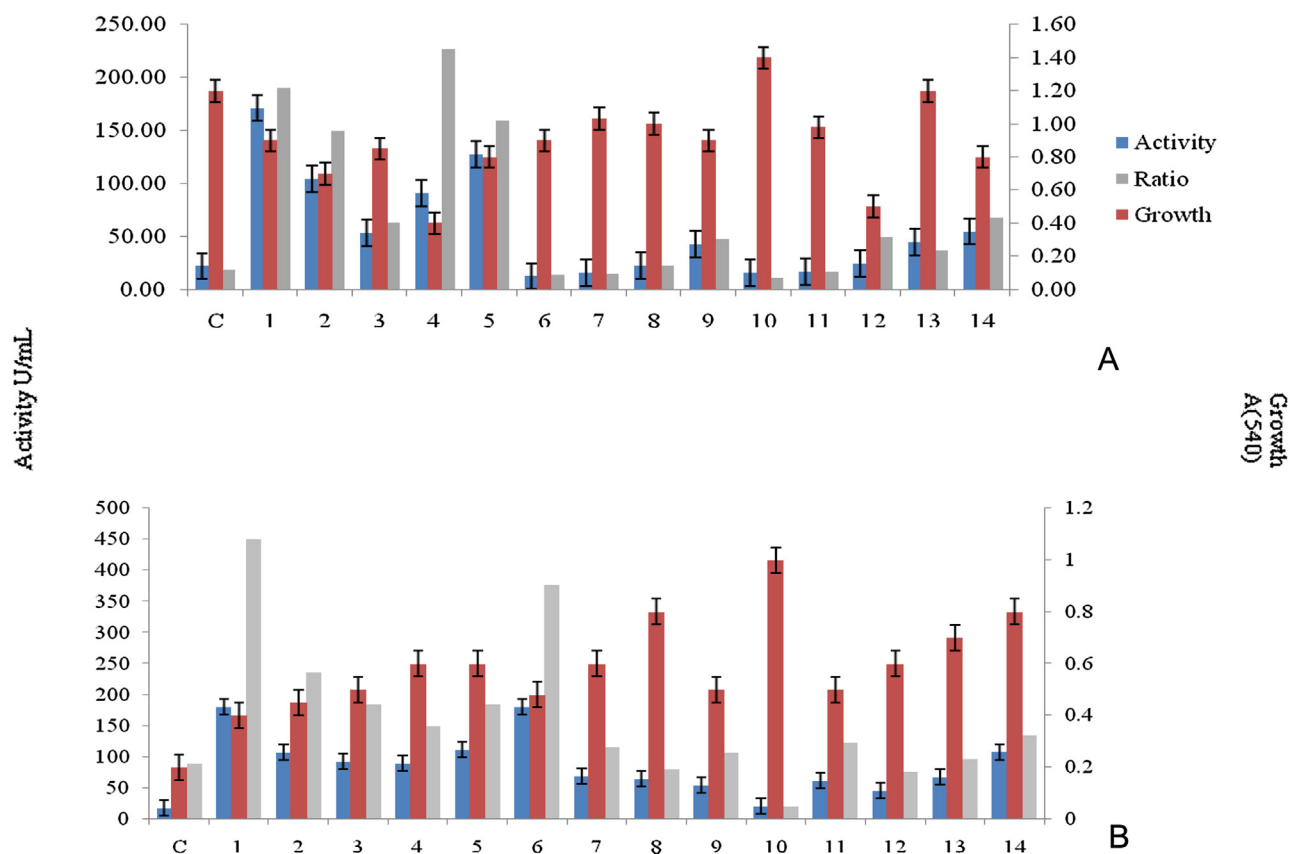
**Fig. 6.** Effect of different amino acids on the growth, ratio and protease production in *Nocardiopsis dassonvillei* OK-18 at pH 11.0 and 30 °C under shake flask (120 rev/min) conditions. (A: with glucose, B: without glucose) (C) Control, (1) proline, (2) proline, glutamine, (3) proline, glutamine, valine, (4) proline, glutamine, valine, isoleucine, (5) proline, glutamine, valine, isoleucine, glutamic acid, (6) proline, glutamine, valine, isoleucine, glutamic acid, threonine, (7) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, (8) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, serine, (9) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, serine, arginine hydrochloride, (10) proline, glutamine, valine, isoleucine, glutamic acid, threonine, tryptophan, serine, arginine hydrochloride, glycine, (11) glycine, alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine, tryptophan, (12) serine, threonine, asparagine, glutamine, tyrosine, cysteine, (13) histidine, isoleucine, methionine, phenylalanine, threonine, tryptophan, valine, (14) alanine, arginine, asparagine, aspartic acid, glutamine, glycine, proline, serine, tyrosine.

Table 1). The protease production in the medium containing only gelatin was $251.19 \pm 3.58$ U/mL. The trends clearly indicated the repression of the enzyme synthesis by different complex carbon and nitrogen sources.

### 3.15. Effect of NaCl on growth and protease production

The actinomycete strain OK-18 was moderately salt tolerant in nature. It could grow well in the range of 0–15% (w/v) NaCl, optimally at 5%. The results revealed that NaCl in the gelatin medium significantly affected the protease production (Supplement Fig. 3). The maximum enzyme production was observed with 5% NaCl (w/v), however, as concentration increased from 5% (w/v) to 15% (w/v), the enzyme production gradually decreased from $251.19 \pm 3.58$ U/mL to 50 U/mL. In the absence of salt, while there was only limited growth, no enzyme production was observed in broth.

### 3.16. Effect of pH on growth and protease production

The effect of pH on the production of protease is displayed in (Supplement Fig. 4). Strain OK-18 produced protease in the wide range of pH from 7.0–11.0. This indicated the alkaliphilic nature of the isolate. The optimum growth of the isolate on agar plate was observed at pH 8–9.0, while optimum production of protease ($251.19 \pm 3.58$ U/mL) occurred at pH 11.0 in the gelatin broth.

### 3.17. Effect of gelatin and different other additives on the growth and protease production

Effect of gelatin was studied with respect to growth and protease production with all other medium components as constant. The maximum protease production was observed at 1.0% (w/v) gelatin. With increasing concentrations of gelatin from 1.0 to 5.0% (w/v), the protease production gradually decreased (Supplement Fig. 5).

Various metal ions and reagents are reported to influence the production and activity of the proteases. Supplement Table 1 summarizes the effect of different additives on protease production by *Nocardiopsis dassonvillei* OK-18. Inorganic phosphate marginally induced the growth with moderate repression of the enzyme production. In the presence of $K_2HPO_4$, $NaNO_3$ and by $MgSO_4$, the enzyme activities were $177.68 \pm 2.87$, $172.62 \pm 0.98$ & $169.82 \pm 3.01$ U/mL, respectively (Supplement Table 1). However, in KCl the enzyme production gradually decreased when compared to control. Over all, the results highlighted that the metal ions did not significantly affect the protease production in this organism.

## 4. Discussion

Proteases catalyse the cleavage of peptide bonds and are present in all living organisms playing important roles in many

physiological functions. The mechanism/s of the regulation of the protease synthesis in prokaryotes is not yet well established. Alkaline proteases comprise around 15% nitrogen and their synthesis is governed by the carbon and nitrogen sources [33].

Although the protease activity can be detected in the early stages of the growth of the organism, the production increases with the growth and reaches to maximum level at the end of the exponential phase, reaching a plateau in the stationary phase. There are number of factors which regulate the proteases production in microbes, including enzyme induction, product inhibition and catalobic repression of the synthesis of enzymes by rapidly metabolizable compounds such as sucrose or glucose or the amino acids [34,35]. While some accounts on the regulation of the proteases synthesis in mesophilic bacteria are available [36,37], reports on similar investigations in haloalkaliphilic bacteria and actinomycetes are rare.

The present study on *Nocardiopsis dassonvillei* OK-18 suggest that the synthesis of extracellular protease occur under the limiting conditions of carbon and nitrogen sources. The influence of amino acids in varied combinations on the protease production in OK-18, a salt-tolerant alkaliphilic actinomycete isolated from coastal Gujarat, India has been described.

The protease synthesis was repressed by leucine and arginine, which apparently favoured growth. The protease production with proline, tyrosine and alanine in minimal medium nearly corresponded to that in complex medium. Therefore they can be a replacement for the complex nitrogen sources, such as malt extract, peptone and yeast extract. Amino acids have earlier been reported to repress the synthesis of alkaline proteases [26,34,35]. The regulation of the protease synthesis in *Bacillus* sp. by alanine has also been reported [38]. Protease synthesis in *Streptomyces clavuligerus* Mit-1was completely inhibited by alanine [26], while in the present report, the OK-18 protease was inhibited by leucine. However, with respect to the effect of methionine, the synthesis of the alkaline protease in OK-18 reflected a corresponding pattern with Mit-1 [26].

Extracellular protease synthesis was strictly regulated in OK-18. The enzyme was synthesized only after the cessation of the active growth in batch culture (Supplement Fig. 1). Repression of the extracellular enzyme synthesis during the early stages of the growth, followed by its de-repression in the late exponential or early stationary phase, is a common feature in bacteria. This phenomenon of the enzyme synthesis has been documented with respect to protease synthesis in haloalkaliphilic actinomycetes [26,27].

*Nocardiopsis dassonvillei strain* OK-18 can utilize a wide variety of substrates. However, the preferred substrates are various amino acids, fermented by the Stickland reactions. Alanine, valine and leucine serve as electron donors while glycine, proline, hydroxyl proline, arginine and orthine act as electron acceptors [39,40]. Variety of amino acids; cysteine, isoleucine, histdine hydrochloride, leucine and arginine hydrochloride repressed the protease synthesis in OK-18, while proline, aspargine, tyrosine, alanine and methionine stimulated the production (Table 1). In other salt tolerant actinobacteria; histidine, methionine and aspartic acid were reported to repress the synthesis of extracellular alkaline protease [26].

The present study revealed that the amino acids in combinations efficiently repressed the protease synthesis (Fig. 6). We examined the effect of varying concentrations of individual amino acids on the protease induction (Table 1). The amino acids were included into the medium with the optimum salt concentration. The di-carboxylic amino acids and their amines, such as aspartic and glutamic acids effectively repressed the protease synthesis. A similar pattern was evident with the amino acids having alcoholic group, such as serine and threonine. The aromatic amino acids

have quite interesting effect on the protease production. With increasing concentrations of tyrosine in the medium, the induction of the alkaline protease synthesis occurred. Non polar aliphatic and small amino acid induced protease synthesis, while isoleucine had negative effect on the enzyme synthesis. The amino acids with hydrocarbon as side chain, such as valine, alanine and methionine induced the protease production, while the basic amino acids, such as histidine and arginine markedly reduced the protease production (Table 1).

De-repression of the protease synthesis occurs under the carbon/energy starvation, oxygen limitation, slow growth rate, high external pH and presence of highly oxidized carbon substrate. These conditions tend to lower the proton motive force and hence decrease the intracellular phosphorylation potential and energy charge [41,42]. In the present study, amino acids, such as alanine, valine, glycine, tyrosine and aspargine apparently de-repressed the protease synthesis. With the increasing concentrations of these amino acids from 0.5 to 1.0% in the minimal medium at pH 11.0, the synthesis of the protease was favoured. The probable reason for the de-repression or positive regulation of the protease synthesis appears to be the slow growth and carbon/energy starvation, as suggested by Kashket (1981).

The protease production with phenylalanine was higher as compared to the tryptophan at that concentration. However, at the same concentration, tyrosine de-repressed and significantly induced the protease production in *Nocardiopsis dassonvillei* strain OK-18, a trend which contradicts the earlier reports for *Bacillus megaterium* [43–45] and *Streptomyces clavuligerus* Mit-1[26]. In another report for *Streptomyces clavuligerus* Mit-1, while growth gradually increased with increasing concentrations of phenylalanine, there was no enzyme production [26].

The Class-3 amino acid, aspartic acid, decreased the protease production in OK-18 at higher concentration of amino acid. Ali [46] reported a similar pattern in *Achlya proliferoides* and *Saprolegnia furcata*, where aspartic acid when supplemented with glucose, at lower concentrations, promoted the protease production as compared to control [46]. However, the enhancement in the enzyme production was reduced with the increasing concentrations of aspartic acid in the medium. A similar trend was observed for two salt tolerant alkaliphilic actinomycetes, where protease production drastically decreased when concentrations of aspartic acid amino acid increased from 1 to 2% (w/v) [27].

The ability to metabolize histidine into ammonia, glutamate and formate or formamide in bacteria is documented [47].The pathway of histidine catabolism is highly conserved among bacteria. In *Vibrio alginolyticus*, the production of the alkaline protease is stimulated by histidine [48].The stimulation of alkaline protease production by histidine and urocanic acid suggests that the hut system may be involved in the regulation of the alkaline protease [48].

In Gram negative bacteria, *Escherichia coli* and *Salmonella typhimurium*, the HUT enzymes are induced by urocanic acid and histidine [49,50]. Control of the HUT operon in these bacteria suggested that urocanic acid, produced from histidine, induces the synthesis of alkaline protease. Interestingly, the HUT pathway in *V. alginolyticus*, a Gram-negative bacterium, is regulated differently as compared to *E. coli* and *S. typhimurium* [49,50]. However, it is similar to the Gram-positive bacterium *Bacillus subtilis* in which histidine (and not urocanic acid) is the inducer of the HUT enzymes [51].

The stimulation of alkaline protease production by histidine and urocanic acid suggests that the histidine utilization (HUT) pathway may be involved in the regulation of the alkaline proteases [52]. In the HUT pathway histidine is converted to glutamic acid via the following intermediates: urocanic acid plus ammonia, 4-imidazolone-5-propionate and N-formimino-glutamate [49]. Bowden et al. [52] investigated the production of

alkaline protease, collagenase and histidine utilization enzymes by *V. alginolyticus* wild type, hutH1 and hutU1 strains. Alkaline protease synthesis was stimulated by histine and urocanic acid in the wild type and mutant strains respectively [52]. Hence, it appears that urocanic acid regulates the alkaline protease synthesis in the minimal medium [48].

The repression of the HUT operon by ammonia and glucose is relieved by histidine. However, at higher concentrations of glucose and ammonia, the histidine effect is significantly reduced. The synthesis of alkaline protease is stimulated in *Bacillus* sp. in a synthetic medium containing glucose and ammonium sulphate [53]. In many gram negative bacteria, histidine is not utilized when glucose is present. In general, histidine degradation is repressed by any easily utilizable carbon source, based on the concept of catabolite repression.

With respect to the repression of the synthesis of alkaline protease observed in strain OK-18, the enzyme synthesis is repressed when glucose is present. In accordance to our study, an alkaline protease was reported to be under the catabolic repression by a number of carbon sources, including glucose and amino acids in *V. alginolyticus* [48]. Interestingly, histidine reversed the repression of the alkaline protease by glucose or ammonium sulphate in minimal medium [48]. In our present report, when histidine was added in the medium along with other amino acids in the presence and absence of glucose, the repression effect was reduced as compared to that with uncharged polar side chain and non polar amino acids.

Stimulation of alkaline protease production in *Streptomyces clavuligerus* strain Mit-1 by histidine in minimal medium containing glucose has been reported [26]. In the present report, the production of protease in histidine and combinations of other amino acids in minimal medium was higher in the absence of glucose (Fig. 6), suggesting that the protease synthesis is regulated via HUT operon.

Among the Class-4 amino acids, histidine and arginine significantly repressed the production of alkaline protease in OK-18. The enzyme production was marginally higher with arginine as compared to histidine which contradicts a previous report of protease in *S. clavuligerus* Mit-1[26]. On a similar note, the significant stimulation of the protease production in minimal medium was reported with arginine in two alkaliphilic actinomycetes OM-6 and OK-5 [27]. Stimulation of protease production at higher concentrations of amino acids has been reported in *Micrococcus* sp. [25] and in three oomycetes (Zoosporic fungi) [46], in synthetic medium with or without glucose. The induction of protease synthesis by various amino acids has also been described in *Aeromonas hydrophilla* [24].

Protease production was significantly stimulated by the non-essential amino acids in the absence of glucose, while the uncharged polar-side chain amino acids completely repressed the synthesis of the alkaline protease. With non-essential amino acids in the minimal medium devoid of glucose, the protease synthesis was higher as compared to the non-polar side chain and essential amino acids. The effect of various combinations of the amino acids on the protease production highlighted that the protease production enhanced with the increasing number of amino acids in the growth medium [26].

The detail mechanism by which protease production is controlled in prokaryotes is not yet well established. The protease synthesis is affected by peptides and other proteinaceus molecules and depending on their concentrations and nature may induce or repress protease synthesis and secretion [21]. As reported earlier, protease production in a salt-tolerant alkaliphilic actinomycete, *Streptomyces clavuligerus* Mit-1, was enhanced with the increasing number of different amino acids in minimal medium [26].

The protease production in many bacteria is inhibited by ammonium salts, and the enzyme production being highly sensitive to the salts. The inhibition of protease production by ammonium salts in various bacteria may be an example of end-product repression, as the proteases generate peptides or amino acids for nitrogen sources [13,54]. Utilization of various amino acids leads to the formation of ammonium salts. It appears that the ammonium salts as nitrogen source would be as effective as amino acids or peptides in suppressing the protease synthesis.

Besides amino acids, other complex nitrogen sources, such as Casamino Acids, are reported to repress protease synthesis in *Bacillus megaterium* [45]. Some strains of *Pseudomonas aeruginosa* use ammonium sulphate as nitrogen source which proved more effective than the combination of several amino acids to repress the protease synthesis [54].

In the present study, maximum enzyme production was obtained with combination of six different amino acids, namely; proline, glutamine, valine, isoleucine, glutamic acid and threonine in the absence of glucose. However, when glucose was present in the minimal medium, the maximum protease production was obtained with combination of proline, glutamine, valine, isoleucine and glutamic acid. As per an earlier report, the maximum protease production in *Streptomyces clavuligerus* Mit-1 was obtained with five different amino acids and glucose in minimal medium [26].

The growth kinetics and alkaline protease production in OK-18 revealed that with increasing concentration of amino acids from 0.5 to 1.0%, the growth of the organism was enhanced, mainly with serine, proline, isoleucine, threonine, phenylalanine, asparagine, leucine, aspartic acid, arginine hydrochloride and methionine. However, with glycine and tyrosine, the growth decreased with increasing concentrations of the amino acids.

In the presence of glucose, combinations of these amino acids promoted the growth. While with the increasing number of uncharged polar side chain amino acids, the growth of the organisms decreased. Interestingly, when glucose was omitted from the minimal medium, the growth restarted with the increasing number of non polar side chain and uncharged polar side chain amino acids (Fig. 6).

Histidine at 1% proved to be the best nitrogen source for the growth of OK-18 in the minimal medium, a trend which corresponded to the actinobacterium *Nocardiopsis* sp. SD5 [55].

As shown in Fig. 5, the OK-18 displayed good growth in aspargine at 1.0% (w/v) and with the increasing concentrations of the amino acid in the minimal medium, the growth increased. Herr [56] reported highest yield of *Aphanomyces cohlioides* mycelium with a combination of low glucose, high asparagine and high methionine [56]. It has also been reported that asparagine stimulated the growth of *Geotrichum candidum* and *Alternaria alternate* [57]. Aspargine has been further stated as the best nitrogen source utilized by *Nocardiopsis* SD5 [55].

Generally, with the amino acids as the nitrogen source in the presence of glucose in basal medium, the growth was stimulated compared to the control that lacked amino acids. Similar observations are reported for the mycelial dry weight in three zoosporic fungal species [46]. Further, when glucose was omitted from the basal medium and amino acids served as the sole source of carbon and nitrogen, the fungal dry weight decreased with the increasing concentrations of the amino acids [46].

In the present study, when glucose was omitted, the enzyme production decreased with the increasing number of amino acids (proline, glutamine, valine and isoleucine) in the medium. Glutamic acid and threonine individually repressed the synthesis of the alkaline protease in minimal medium. However, when both amino acids were added along with other amino acids and in the absence of glucose, the protease production was enhanced. The cell

growth increased with the increasing number of amino acids which served as carbon and nitrogen.

A limited growth of *Aphanomyces euteiches* in the absence of glucose is reported by Papavizas and Davey [58]. The nitrogen sources in the synthetic media were poorly utilized as carbon source by *Aphanomyces euteiches* [58]. Similarly, the extracellular protease production in *Aphanomyces proliferoides* in the synthetic medium supplemented with different concentrations of amino acids without glucose was gradually suppressed [46].

*S. ferax* utilized alanine, leucine, tyrosine and phenylalanine more rapidly in the absence of glucose [59], a study which corresponds to our findings in this report. In *B. stearothermophilus* F1, only limited growth and enzyme production was observed with cysteine, leucine, arginine and glycine used as the sole nitrogen source [60]. With uncharged polar side chain amino acids, however, the synthesis of the protease in OK-18 was promoted when glucose was omitted from the basal medium.

Some actinomycetes are reported to grow slowly under high salt and alkaline conditions [26,27]. The enzyme production has been reported to begin in early log followed by a drastic increase during the late growth phase of *Streptomyces* sp. D1 [61]. OK-18 produced protease maximally during the late exponential to early stationary phase of the growth which draws similarity with the previously reported trends in *Nocardiopsis alba* OK-5 [27,28] and *Streptomyces clavuligerus* Mit-1 [11,26].

The size of the inoculums affects the enzyme production in microorganisms and plays a crucial role in the fermentation process [32]. Elibol and Moreira [32] reported a 2.5% (v/v) inoculum load as optimum for the production of an alkaline protease in a marine bacterium *Teredinobacter turnirae*. Enzymes were susceptible to mechanical force and often denaturation occurs above the critical values. The maximum protease production was observed at 120 rpm and with 3% (v/v) inoculum load for the strain OK-18, suggesting a similarity with a previously reported marine bacterium *Teredinobacter turnirae*. A further increase in the agitation speed did not affect protease production by *T. Turnirae* [32]. On the other hand, higher inoculum sizes are likely to reduce the dissolved oxygen, leading to enhanced competition for the nutrients in the medium [60].

Further, the protease production heavily depends on the nitrogen and carbon sources in the medium, where both play regulatory roles in the enzyme synthesis. Protease production is under the repression by various nitrogen sources [62].

The present study and those reported earlier suggest that the microorganisms have different preferences of organic and inorganic nitrogen sources for the growth and protease production. The present report on the repression of the protease suggests catabolic effect by different carbon and nitrogen sources. While glucose in the medium promotes bacterial growth, it negatively affects the protease synthesis. Glucose at higher concentrations inhibits the alkaline protease production in alkaliphilic actinomycete [10]. In *Actinopolyspora* sp. VITSDK2 from a marine saltern, the growth and protease activity was enhanced by galactose and glucose [63]. Similarly, fructose induced protease production has been reported in *Streptomyces* sp. DP2 [64]. Strain OK-18 has a preference for fructose than glucose for protease production, which correspondence to an earlier report by Bajaj and Sharma [64].

Complex organic nitrogen sources such as yeast extract and peptone are rich in amino acids and short peptides and bring enzyme repression at higher concentrations. The data in this report highlighted that while yeast extract promoted growth of OK-18, the synthesis of the protease was inhibited. Similarly, with the increasing concentrations of peptone and malt extract, the production gradually decreased, reflecting a trend earlier reported for *B. firmus* [21]. The effect of various nitrogen sources on L-

glutamate oxidase synthesis in *Streptomyces cremeus* 510 MGU is described [65].

In *B. stearothermophilus* F1, the enzyme production gradually decreased beyond a threshold level of peptone [46]. Patel et al. [66] reported that in *Bacillus* sp. isolated from the coastal Gujarat, (India), gelatin and casamino acids served as the best nitrogen sources enhancing the growth and protease production up to a threshold level [66].

OK-18, a salt tolerant actinomycete, produced protease optimally at 5% (w/v) NaCl, while it grew up to 15% (w/v) NaCl. Similarly, a haloalkaliphilic actinomycete strain Mit-1, can grow up to 10% (w/v) NaCl, but optimally produced protease at 5% NaCl (w/v) [11]. A similar trend was also evident in *Salinicoccus alkaliphilus* sp.nov., halo-alkaliphiliccoccus isolated from Mongolia, which could grow over a wide range of NaCl concentrations (0–25%) with optimum at 10% (w/v)[67]. The secretion of the alkaline protease in OK-18 was not directly linked with the growth of the organism. The growth of the halotolerant bacteria is affected by the polar lipid composition of the cell membranes, and an increased salt concentration creates change in the lipid environment that leads to the decreased growth and reduced enzyme production [68]. The high salt tolerance of the halophilic enzymes is a characteristic which may lead to a number of future applications [2]. Further, the halophilic proteins compete effectively with salts for hydration, a property that may account for resistance against other extreme environments, such as organic solvent tolerance. Novel halophilic biomolecules may also be used for specialized applications [69].

Medium pH strongly affects enzymatic processes and transport of several species across the cell membrane. A change in pH alters the acid-base equilibria and fluxes of various nutrients, inducers and growth factors between the abiotic and biotic phase [21]. The optimum pH for growth and protease production varies in actinomycetes as evident from our present study and reports in the literature [70–72]. Further, *Streptomyces* spp. from soil [73] and other alkaliphilic actinomycetes, *Nocardiopsis dassonvillei* [74,75] were earlier reported to produce alkaline proteases in broader range of the alkaline pH. Alkaliphilic actinomycete isolated from sediment samples of the Izmir Gulf, Turkey required pH of 8.0–11.0 for growth [76]. An alkaliphilic *Microbacterium* sp. from the Lake Arenguadie (Ethiopia) produced protease over a broad pH range with the optimum at 9.5–11.5 [77]. Thus it appears that the optimum pH range of 9.0–10.0 is common in the alkaliphilic and haloalkaliphilic organisms for the growth and protease production [10,11,26–28].

The effect of gelatin and other additives was investigated in OK-18 with respect to growth and protease production, where maximum enzyme was produced with 1% (w/v) gelatin, while maximum growth occurred with 5% gelatin. A corresponding trend of the effect of geletin has been earlier reported in *Streptomyces clavuligerus* Mit-1, an alkaliphilic and salt- tolerant actinomycete [26]. According to another report, gelatin stimulated the enzyme production in two strains of the haloalakliphilic actinomyetes [27].

## 5. Conclusion

The catabolic repression of the industrially important enzymes poses serious problems in the submerged fermentation. In order to bypass the regulatory barrier, fed- batch or continuous cultures and the mutants resistant to the catabolic repression can be explored. However, these approaches are not cost effective. In this study, we focused on the regulation of the protease synthesis in a salt tolerant alkaliphilic actinomycete, *Nocardiopsis dassonvillei* OK-18. Carbon and nitrogen sources were the most significant factor affecting the production of protease in this strain. The protease production was regulated by different combinations of

amino acids in the presence or absence of glucose. The production of the enzyme appeared delinked with the cell growth. The saline habitat used for the isolation of the actinomycetes has not been systematically explored for the biocatalytic potential of the microbes, particularly the actinomycetes. The regulation of the synthesis of protease in actinomycetes from these habitats, therefore, highlights the significance of the study.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2016.10.004.

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