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Improving α-amylase production by *Bacillus licheniformis* ATCC 6346 with local nitrogen sources and amino acids supplementations

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ABSTRACT

Microbial α-amylase is highly demanded industrial enzyme in various sectors such as pharmaceuticals, food, textile and detergents, etc. In this study production of α-amylase by Bacillus licheniformis ATCC 6346 was investigated with locally available nitrogen sources such as mustard, sesamum and coconut seed cake powders instead of peptone (6 gL^{-1}). The highest α-amylase activity (32.95 UmL^{-1}) was produced in the medium containing mustard powder while negligible amount (0.69 UmL^{-1}) was obtained in the medium with coconut seed cake powder. Among the defatted local nitrogen sources, highest α-amylase activity (58.14 UmL^{-1}) was produced in the medium with defatted mustard seed cake powder (18 gL^{-1}) and least activity (29.61 UmL^{-1}) was produced in presence of defatted coconut seed cake powder (24 gL^{-1}). Sesamum seed cake powder (18 gL^{-1}) and coconut seed cake powder (24 gL^{-1}) containing media were supplemented with different amino acids or their mixture equivalent to the present in mustard seed cake (18 gL^{-1}) powder. Supplementation of 0.0147 and 0.10801 gL^{-1} tryptophane respectively to defatted sesamum (18 gL^{-1}) and coconut seed cake powder (24 gL^{-1}) containing media increased the production of α-amylase to 57.42 and 58.26 UmL^{-1}, which were almost equalent to that produced in defatted mustard seed cake powder.

Keywords: α-Amylase, *Bacillus licheniformis*, seed cake powder, local nitrogen sources, Peptone.

INTRODUCTION

α-Amylases are important industrial enzymes [1] and rank first in terms of commercial uses [2, 3]. α-Amylases (EC 3.2.1.1, 1,4-α-D glucanohydrolase, endoamylase) hydrolyse starch, glycogen and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkages. Amylases are among the most important enzymes in present-day biotechnology, and are universally distributed throughout the plant, animal and microbial kingdom. Microbial production of α-amylases is expensive due to the highest-cost nitrogen sources. The regular use of peptone based fermentation media is not commercially viable for industries. For efficient

commercial production, a continuous effort is being made to find cheaper substrate sources. Available carbon and nitrogen sources are the decisive factors in the optimum production of enzymes, and these differ very much from substrate to substrate [4]. Several oil seed cakes, because of their abundant availability and low price, are used as cattle feed [5], fertilizer [6], and in rare cases after proper processing, as food for humans [7]. Therefore the oil seed cakes such as sesamum seed cake, coconut seed cake which are available in the local market at Jaffna after the oil extract could be used as carbon and nitrogen sources. The present study was planned to use sesamum and coconut oil seed cakes which are available locally to produce α-amylase by *Bacillus licheniformis* ATCC 6346. These seed cakes were compared with the mustard seed cake, which has been reported as a good source for α-amylase production [4].

MATERIALS AND METHODS

Materials

Sesamum and coconut seed cakes were obtained from local market in Jaffna and powered with a domestic grinder. Mustard seed cake powder was prepared in the laboratory at Department of Biochemistry, Faculty of Medicine, University of Jaffna by crushing the seeds purchased in the local market with motor and pestle, air drying and powdering with a domestic grinder.

Strain

Bacillus licheniformis ATCC 6346 from Heriot-Watt University U.K was used in this study.

Growth media and α-amylase production

The bacteria was activated in activation medium [(gL^{-1}) nutrient broth, 25.0 and soluble starch 3.0 at pH 7.0]. To the fermentation medium [(gL^{-1}) soluble starch, 4.0; $(NH_4)_2SO_4$, 5.0; peptone, 6.0; $FeCl_3$, 0.01; $MgCl_2.6H_2O$, 0.01; $CaCl_2.2H_2O$, 0.01; KH_2PO_4, 4.0 and K_2HPO_4, 7.5 at pH 7.0] 12h old inoculum was inoculated and incubated at 42^oC.

Effect of different local and commercial nitrogen sources on α-amylase production

Different commercial nitrogen sources (6 gL^{-1}) such as tryptone or yeast extract or casamino acid or local nitrogen sources (6 gL^{-1}) such as mustard seed cake powder or sesamum seed cake powder or coconut seed cake powder was added to the fermentation medium instead of peptone (6 gL^{-1}) and the α-amylase activity produced was monitored [8].

Effect of different defatted local nitrogen sources on α-amylase production

Defatted local nitrogen sources (6 gL^{-1}) such as mustard seed cake powder or sesamum seed cake powder or coconut seed cake powder was added to the fermentation medium instead of peptone (6 gL^{-1}) and α-amylase activity produced was monitored.

Optimization of the amount of defatted local nitrogen sources on α-amylase production

Different amounts (from 2.0 to 30 g) of completely defatted seed cake powder of mustard, sesamum and coconut were added to the fermentation medium instead of peptone (6 gL^{-1}) and α-amylase activity produced was monitored.

Effect of different amino acids supplementation to either defatted sesamum seed cake powder or defatted coconut seed cake powder on α-amylase production

Sesamum seed cake powder (18 gL^{-1}) was supplemented with either tryptophan (0.0147 gL^{-1}) or threonine (0.0514 gL^{-1}) or leucine (0.0370 gL^{-1}) or lysine (0.2040 gL^{-1}) or valine (0.0326 gL^{-1}) or histidine (0.0330 gL^{-1}) or alanine (0.0179 gL^{-1}) or glutamic acid (0.0560 gL^{-1}) or proline (0.2370 gL^{-1}) or the mixture of all the amino acids in the above said amount. Sesamum seed cake powder

(18 gL^{-1}) containing medium without the supplementation of amino acids was used as control and mustard seed cake powder (18 gL^{-1}) containing medium was used as reference medium.

The above same procedure was repeated with coconut seed cake powder (24 gL^{-1}) by supplementing with either tryptophan (0.10801 gL^{-1}) or histidine (0.1476 gL^{-1}) or valine (0.2246 gL^{-1}) or lysine (0.3060 gL^{-1}) or glutamic acid (0.8790 gL^{-1}) or proline (0.4073 gL^{-1}) or glycine (0.2386 gL^{-1}) or isoleucine (0.0588 gL^{-1}) or phenylalanine (0.1784 gL^{-1}) or serine (0.1774 gL^{-1}) or leucine (0.3128 gL^{-1}) or alanine (0.2081 gL^{-1}) or these amino acids mixture.

RESULTS AND DISCUSSION

Effect of different local and commercial nitrogen sources on α-amylase production

An attempt was made to identify cheap nitrogen sources for α-amylase production and hence different commercially and locally available nitrogen sources were selected for the production of α-amylase. At 48 h, α-amylase production in the media containing tryptone was highest (43.48 UmL^{-1}) while that in control medium which contained peptone was 44.43 UmL^{-1} (Table I). Lipid, total nitrogen, carbohydrate and most of the amino acid contents of peptone and tryptone were almost same (The OXOID Manual) and this could be the reason for the same amount of enzyme production while the total nitrogen contents in yeast extract and casamino acids were less than that in peptone (The OXOID Manual). Tryptone and peptone have been reported to support α-amylase secretion by *B.stearothermophilus* [9] and *B. licheniformis* [10]. Salt content present in casamino acids was higher than that of peptone (The OXOID Manual). The results indicated that the total nitrogen content of the commercial nitrogen sources has influenced α-amylase production.

In the media containing local nitrogen sources, the α-amylase activities obtained were less than that in the control medium (Table I). All the locally available nitrogen sources selected for the study contained less amount of total nitrogen than that in peptone (Table I). The protein content of mustard and sesamum seed cake powder was very close to each other, while α-amylase production was higher in presence of the former (32.95 UmL^{-1}) than in later. The variations could be due to the amino acid contents of the proteins or be due to the fat contents of these sources. Therefore the fat present in the local nitrogen sources were removed and the α-amylase production was studied. Highest amount of total sugar was present in coconut seed cake powder while the least amount of total sugar was found in sesamum seed cake powder (Table I). Therefore the amount of total sugar cannot be correlated to α-amylase production under the conditions studied in this experiment.

Effect of different defatted local nitrogen sources on α-amylase production

α-Amylase activities produced in defatted mustard, sesamum and coconut seed cake powder containing media were higher than those in the media which contained respective seed cake powder with fat (Table II). This indicated that the fat present in these sources might have inhibited α-amylase production. In the absence of fat, the total nitrogen content of sesamum and mustard seed cake powder were almost same and the α-amylase production were also same (Table II). The total nitrogen content of coconut seed cake powder was lowest and the α-amylase production was also lowest (Table II).

Table I: Effect of different commercially available and local nitrogen sources ($6gL^{-1}$) on □-amylase production by *B.licheniformis* ATCC 6346 at $42^{o}C$, 100rpm and at 48h. □-Amylase activity was determined at $85^{o}C$ and pH 7.0 (0.01M phosphate buffer) using $20gL^{-1}$ starch as substrate and at 5min of incubation.

Nitrogen sources in 6g sources(g)	**Total nitrogen in 6g sources (g)**	**Total protein in 6g sources (g)**	**Total carbohydrate in 6g sources (g)**	**Total fat activity □-amylase (UmL^{-1})**	**□-Amylase activity (%)**	**Relative**
Peptone	0.7980	4.9875	-	-	44.43	100
Tryptone	0.7620	4.7625	-	-	43.48	97.86
Yeast extract	0.5880	3.6750	-	-	36.07	81.18
Casamino acids	0.6000	3.7500			39.83	89.64
Mustard seed cake powder	0.3162	1.9770	0.2646	1.7952	32.95	74.16
Sesamum seed cake powder	0.3426	2.1432	0.2406	0.8154	21.11	47.51
Coconut seed cake powder	0.1461	0.9132	0.3936	0.6564	0.691	1.55

Protein content of Mustard, Sesamun and coconut seed cake powders were 32.95, 35.72 and 15.22 (%) respectively.
Sugar content of Mustard, Sesamun and coconut seed cake powders were 4.41, 4.01 and 6.56 (%) respectively.
Fat content of Mustard, Sesamun and coconut seed cake powder weres 29.92, 13.59 and 10.94 (%) respectively.

Table II: The □-amylase production by *B.licheniformis* ATCC 6346 in the media containing 6gL^{-1} of local nitrogen sources in presence and absence of fat at 42^{o}C, 100rpm and at 48h. □-Amylase activity was determined at 85^{o}C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} starch as substrate and at 5min of incubation.

Local nitrogen sources			Nitrogen sources			defatted nitrogen sources		
	Total nitrogen in 6g source (g)	**Total CHO in 6g source (g)**	**□-Amylase activity (UmL^{-1})**	**Total nitrogen in 6g source (g)**	**Total CHO in 6g source (g)**	**□-Amylase activity (UmL^{-1})**		
Peptone	0.798	-	44.43	-	-	-		
Mustard seed cake powder	0.316	0.2646	32.95	0.410	0.343	46.01		
Sesamum seed cake powder	0.342	0.2406	21.11	0.389	0.273	46.79		
Coconut seed cake powder	0.146	0.3936	0.691	0.162	0.436	14.74		

Protein content of defatted Mustard, Sesamun and coconut seed cake powders were 42.8, 40.6 and 16.8 (%) respectively.
Sugar content of defatted Mustard, Sesamun and coconut seed cake powders were 5.7, 4.5 and 7.2 (%) respectively.

In presence of defatted Mustard and Seamum seed cake powder, higher activity of α-amylase was obtained than in peptone containing medium, while the total nitrogen in 6g of Mustard and Seamum seed cake powder was less than 6 g of peptone (Table II). Therefore the total nitrogen content is not the only factor, which influences the α-amylase production. Among the defatted local nitrogen sources 6 g of coconut seed cake powder contained highest amount of total sugar (0.3936 g) than other defatted local nitrogen sources but in this medium less enzyme production (14.74 UmL^{-1}) was obtained than in the media with other defatted nitrogen sources (Table II). Therefore the total sugar present in the local nitrogen sources also not the only factor, which influences the α-amylase production.

Optimization of the amount of defatted local nitrogen sources on α-amylase production

Coconut and sesamum seed cake powder preparations were abundantly available in local market at very low price. Mustard seed cake powder was prepared because it was reported that high protein content and a promising source for α-amylase production [4]. This experiment was carried out to find the suitable concentration of the seed cake powder which can produce maximum amount of the enzyme.

When the amount of defatted mustard seed cake powder in the media was varied from 2.0 to 30.0 gL^{-1} while the other components of the media were kept constant, α-amylase produced in presence of 18.0 gL^{-1} defatted mustard seed cake powder was the highest (58.14 UmL^{-1}, Table III) and 1.5 times higher than that produced in the control medium (6 gL^{-1} peptone). Thus increase in total nitrogen and sugar contents do not simply influence the α-amylase production. When the concentration of defatted sesamum seed cake powder in the medium was changed, highest α-amylase activity (56.64 UmL^{-1}, Table IV) was obtained in the medium containing 18 gL^{-1} sesamum seed cake powder, and was 1.4 fold higher than that in the control medium with 6 gL^{-1} peptone.

α-Amylase activity produced in the medium containing 24 gL^{-1} defatted coconut seed cake powder was the highest (29.61 UmL^{-1}) at 48 h (Table V). α-Amylase production in coconut seed cake powder containing medium was improved when the fat was removed. This indicated that the residual fats in coconut seed cake powder have some adverse effect on α-amylase production. Coconut seed cake is generally fed to animals and finds no other application [11].

The α-amylase production in defatted mustard seed cake powder was higher than that in all the media with defatted local nitrogen sources. Mustard seed cake powder contained lower level of sugar and higher level of protein than coconut seed cake powder (Table II), and the α-amylase activity produced was 56.64 UmL^{-1}. From the results it can be concluded that the Carbon (Total) and Nitrogen (Total) are not the only factors which influence the α-amylase production by *B.licheniformis* ATCC 6346. Among the defatted local nitrogen sources, defatted coconut seed cake powder containing media were inferior for α-amylase production. The total nitrogen content of 24 g coconut seed cake powder (0.65 g) was the least among other local nitrogen sources gave highest α-amylase activity.

This study on the effect of local nitrogen sources on α-amylase production by *B.licheniformis* ATCC 6346 is of interest because the local nitrogen sources can completely replace peptone except coconut seed cake powder. Peptone is costly and impractical for commercial production of the enzyme.

Table III: α-Amylase production by *B.licheniformis* ATCC 6346 in media containing different amounts of defatted mustard seed cake powder. The total nitrogen and sugar contents of the medium related to different amounts taken. α-Amylase activity was determined at 85°C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} starch as substrate and at 5min of incubation.

Mustard seed Cake powder (g)	Total nitrogen (g)	Total sugar (g)	α-Amylase Activity (UmL^{-1})	α-Amylase Relative activity (%)
Control (6gL^{-1} peptone)	0.798	-	37.98 ± 3.48	100
2	0.14	0.11	41.66	120
6	0.41	0.34	46.01	133
10	0.68	0.57	51.48	149
14	0.96	0.79	52.59	152
18	1.23	1.03	58.14	168
24	1.64	1.36	57.12	165
30	2.05	1.71	54.56	158

Table 1V: α-Amylase production by *B.licheniformis* ATCC 6346 in media containing different amounts of defatted sesamum seed cake powder. The total nitrogen and sugar contents of the medium related to different amounts taken. α-Amylase activity was determined at 85°C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} starch as substrate and at 5min of incubation.

Sesamum seed cake powder (g)	Total nitrogen (g)	Total sugar (g)	α-Amylase Activity (UmL^{-1})	α-Amylase Relative activity (%)
Control (6gl^{-1} peptone)	0.798	-	37.98 ± 3.48	100
2	0.13	0.09	36.94	107
6	0.39	0.27	46.79	135
10	0.65	0.45	46.51	134
14	0.91	0.63	51.18	148
18	1.17	0.81	56.64	164
24	1.56	1.08	50.80	147
30	1.95	1.35	44.51	129

The coconut and sesamum seed cakes are by-products of oil industries, very cheap, readily available, natural, and fairly acceptable nutrient sources for *B.licheniformis* ATCC 6346. Further the defatted local nitrogen sources remarkably enhanced the production of thermostable α-amylase from *Bacillus licheniformis* ATCC 6346. Among the other nutrients amino acids might have quantitatively and qualitatively varied in the protein and might have influenced the enzyme production. Amount of amino acids present in peptone was higher than that present in defatted mustard seed cake powder except tryptophan (The OXOID Manual, 12). This excess amount of tryptophan present in defatted mustard powder could be the reason for improved α-amylase production in mustard seed cake powder containing medium than peptone containing medium. Hence the effect of amino acids on the production of α-amylase was investigated in the following experiment.

Effect of different amino acids supplementation to defatted sesamum seed cake powder or defatted coconut seed cake powder on the production of α-amylase

Since α-amylase production was highest in defatted mustard seed cake powder than in all other

media considered, it was thought that the amino acid contents of the proteins of mustard seed cake powder might have influenced the α-amylase production. Hence the difference in the amino acid contents between the defatted sesamum seed cake powder and defatted mustard seed cake powder were considered and the sesamum seed cake powder was supplemented with the respective amounts of the amino acids separately to the sesamum seed cake powder (18 gL^{-1}) containing medium. Here the amino acid supplementation was calculated based on the difference between those present in 18 gL^{-1} mustard powder and in 18 gL^{-1} of sesamum seed cake powder [12, 13].

Table V: α-Amylase production by *B.licheniformis* ATCC 6346 in media containing different amounts of defatted coconut seed cake powder. The total nitrogen and sugar contents of the medium related to different amounts taken. α -Amylase activity was determined at 85°C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} starch as substrate and at 5min of incubation.

Coconut seed cake powder(g)	Total nitrogen (g)	Total sugar (g)	α-Amylase Activity (UmL^{-1})	α-Amylase Relative activity (%)
Control (6gL^{-1} peptone)	0.798	-	37.98 ± 3.48	100
2	0.05	0.14	10.92	31
6	0.16	0.43	14.74	42
10	0.27	0.72	15.85	45
14	0.38	1.00	21.52	62
18	0.48	1.29	25.02	72
24	0.65	1.78	29.61	85
30	0.81	2.16	27.36	79

Among the different amino acids supplemented in presence of 18 gL^{-1} of sesamum seed cake powder, tryptophan increased the production of α-amylase followed with lysine (Table VI).But the enzyme production was decreased by threonine, leucine, valine, histidine and proline when compared with the control medium which contained 18 gL^{-1} sesamum seed cake powder (Table VI). In the amino acids mixture supplemented medium 55.58 UmL^{-1} α-amylase activity was obtained at 48 h. This was 96 % of that obtained in the mustard seed cake powder containing medium and 109 % of that obtained in control medium (which containing 18 gL^{-1}sesamum seed cake powder).

Almost same amount of α-amylase activity was obtained in the defatted sesamum seed cake powder (18 gL^{-1}) containing media supplemented with tryptophan (57.42 UmL^{-1}) and 18 gL^{-1} mustard powder (57.74 UmL^{-1}). Therefore supplementation of tryptophan to defatted sesamum seed cake powder has successfully increased the production of α-amylase. In defatted sesamum seed cake powder containing medium supplemented with lysine maximum α-amylase activity produced was 106 % of that obtained with control medium. These results indicated that either tryptophan or lysine supplementation to sesamum seed cake powder gave positive effect on the production of α-amylase. Therefore either tryptophan or lysine present in mustard seed cake powder (18 g) could be the cause for increased production of α-amylase in mustard seed cake powder (18 g) containing medium. Among the defatted local nitrogen sources α-amylase production was least in defatted coconut seed cake powder containing medium. Therefore the effect of amino acids on the production of α-amylase from *Bacillus licheniformis* ATCC 6346 in 24 g defatted coconut seed cake powder containing medium was studied. Here the amino acid

supplementation was calculated based on the difference between those present in 18 gL^{-1} mustard powder and in 24 gL^{-1} of coconut seed cake powder [12, 14]

Table VI: Effect of individual and mixture of all the amino acids supplementation on the production of α-amylase by *B.licheniformis* ATCC 6346 in media containing 18gL^{-1}defatted sesamum seed cake powder as nitrogen source at 42°C and at pH 7.0 while shaking at 100rpm and 48h. α-Amylase activity was determined at 85°C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} starch as substrate and at 5min of incubation.

Amino acid	α-Amylase Activity (UmL^{-1})	Relative activity (%)
Control (18gL^{-1} sesamum)	50.99	100
Tryptophan	57.42	112
Threonine	39.34	77
Leucine	34.51	67
Lysine	54.19	106
Valine	42.48	83
Histidine	44.27	86
Alanine	48.31	94
Glutamic acid	47.31	92
Proline	36.40	71
Mixture (All aminacids)	55.58	109
Mustard (18gL^{-1})	57.74	113

Mustard seed cake powder (18gL^{-1}) containing medium was used as reference medium

Production of α-amylase was increased by supplementing the defatted coconut seed cake powder (24 gL^{-1}) with different amino acids and the mixture of these amino acids at 48hours (31.36 UmL^{-1}, Table VII). The α-amylase activity obtained in the medium supplemented with tryptophan (58.26 UmL^{-1}) was highest and it was 102 % higher than that in 18 gL^{-1} defatted mustard seed cake powder containing medium (56.78 UmL^{-1}). However α-amylase production in the tryptophan supplemented medium was 186 % of that in the defatted coconut seed cake powder. Thus tryptophan supplementation has increased the production of α-amylase. Therefore these results indicated that tryptophan can influence the production of α-amylase by *B.licheniformis* ATCC 6346. Alanine and lysine supplementation also has improved α-amylase production above 50 UmL^{-1} and these were respectively 173 and 163 % higher than that obtained in the control medium. Therefore the supplementation of tryptophan, alanine and lysine to equalize the amount of these amino acids present in mustard powder (18 g) could increase the production of α-amylase in coconut seed cake powder (24 g) containing medium (Table VII). Supplementation of the mixture of all amino acids to coconut seed cake powder increased the production of α-amylase by 147 % than in control medium. This experiment has indicated that the supplementation of local seed cake powder with amino acids could improve α-amylase production. Some amino acids are believed to be absolutely required for the growth of many thermophilic *Bacillus strains* [15]. However the α-amylase production in the medium supplemented with amino acids mixture (46.11 UmL^{-1}, Table VII) was less than that produced in the medium supplemented with tryptophan (58.26 UmL^{-1}) or lysine (50.85 UmL^{-1}) or alanine (54.26 UmL^{-1}). This could be due to the inhibiting effect of other amino acids present in the mixture.

Table VII: Effect of individual and mixture of all the amino acids supplementation on the production of α-amylase by *B.licheniformis* ATCC 6346 in media containing 24gL^{-1} defatted coconut seed cake powder as nitrogen source at 42^{o}C and at pH 7.0 while shaking at 100rpm and 48h. α-Amylase activity was determined at 85^{o}C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} starch as substrate and at 5min of incubation.

Amino acid	α-Amylase	
	Activity (UmL^{-1})	Relative activity (%)
Control (24gL^{-1} coconut)	31.26	100
Tryptophan	58.26	186
Leucine	48.36	154
Valine	44.01	140
Histidine	45.16	144
Alanine	54.26	173
Glutamic acid	43.21	138
Proline	45.16	144
Isoleucine	40.86	130
Lysine	50.85	162
Phenylalanine	41.35	132
Glycine	40.11	128
Serine	44.26	141
Mixture (All aminacids)	46.11	147
Mustard (18gL^{-1})	56.78	181

Mustard (18gL^{-1}) containing fermentation medium was used as reference medium

CONCLUSION

It is evident from this study that oil seed cakes can serve as ideal fermentation sources for obtaining high yields of α-amylase production by *Bacillus licheniformis* ATCC 6346. The effect of oil seed cakes on α-amylase production by this strain is of interest because the oil seed can completely replace the peptone which is costly for the commercial production of α-amylase. Mustard and Sesamum can completely replaced peptone which is costly and impractical for the commercial production of the enzyme. The local sources which are rich in tryptophan and lysine could be supplemented to the nitrogen sources to increase α-amylase production instead of the Analar grade amino acids. We cannot conclude that the tryptophan is the only factor that has increased α-amylase production in mustard seed cake powder containing medium. And some factors (such as minerals and vitamins) other than amino acids present in mustard seed cake powder could also be the cause for this increase.

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