

Original Research Article

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Process Parameter Optimization for Asparaginase Production from *O. intermedium*: A Potential Candidate for Acrylamide Mitigation

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ABSTRACT

Asparaginase (asn) is a promising candidate for the obviation of acrylamide formation in baked and fried food stuffs by pre amidohydrolase of free asparagine present. In this study optimized the medium composition for asparaginase production from *Ochrobactrum. intermedium* using one variable at a time approach. Maximum yield of asparaginase was recorded with batch time 18 h, inoculum size 2%, pH 7.0, and temperature 40°C. Lactose, yeast extract, magnesium ions, asparagine were found to be best carbon, nitrogen, mineral ions and inducer respectively for asparaginase production. An overall 5.045 (13.956±0.07 IU/ ml) fold increased as compared to initial un optimized activity of 2.766 IU/ml was obtained. These results suggested that selected parameter can be promisingly enhanced the asparaginase production. The further attempts are underway for validation of significant factors by statistical approach to influence asparaginase yield from the strain for acrylamide inhibition.

Keywords

Process,
Parameter,
Optimization and
O. intermedium.

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Introduction

Acrylamide (C₃H₅NO) is a toxic aliphatic amide compound, usually formed industrially from the hydration of acrylonitrile. The occurrences of acrylamide during heating above 120°C in commonly consumed starch based foods are leading to great concern worldwide since 2002 (Sangavi *et al.*, 2016). It has variety of adverse effects in animals and humans such as neurotoxicity, reproductive toxicity, genotoxicity, and classified as Group 2A carcinogen by international agency for research in cancer (Xu *et al.*, 2014). Maillard reaction in the presence of asparagine, is the major reaction pathway of acrylamide formation in food(s)

(Anese *et al.*, 2009). Food industry faces challenge to modify the process parameters for acrylamide reduction apart from the available techniques, asparaginase pretreatment claimed to be promising technological intervention which significantly reduced acrylamide formation (Zuo *et al.*, 2015). Asparaginase (asparagine amidohydrolase, E.C.3.5.1.1) catalyzes the hydrolysis of asparagine key precursor of acrylamide into aspartic acid and ammonia with maintaining the sensory characteristics properties of final product (Pedreschi *et al.*, 2014). They widely exist in all living organisms, including microorganisms, plants

and animals but due to its tedious extraction procedure, microorganisms have been found to be more efficient and cost effective Hendriksen *et al.*, (2009). The selection and production of extracellular asparaginase be preferred over other intracellular enzyme because of having easy extraction, protease deficient compartment and relatively free from endotoxins which affect in minimization of adverse effects (Pradhan *et al.*, 2013) Each bacterial species has its own particular nutritional and environmental requirement and there is no standard medium has been developed for the optimum production of asparaginase from bacterial species (Chidambaram *et al.*, 2009).

In commercial practice, the optimization of the medium is required to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation (Rehman *et al.*, 2005). So, the optimization of medium components and cultural parameters is the primary task for biological process. The traditional one factor at-a-time optimization strategy gives the individual effects of medium components, it can be seen on a graphically representation without the need to revert to statistical analysis. The potato chips pre-treated with the asparaginase was found to inhibit 53% of acrylamide formation against untreated slices (data not shown). Hence, it is desirable to find ways to produce asparaginase in higher yield, to procurement of large quantity of material required for acrylamide reduction. With the above perspective in view, an investigation was carried out in step wise optimization strategy with reference to process parameter for optimization of asparaginase production from *O. intermedium*.

Materials and Methods

L-asparagine and all other chemicals used were procured from Himedia, Mumbai, India.

Reagents used in the experiments were of analytical grade and used according to the specification provided by the manufacturer.

Culture conditions

The asparaginase producing strain, *O. intermedium* (NCBI accession no: KT157596) was obtained from Bacterial Germplasm Collection Centre (BGCC#2407), Bacteriology Laboratory, Department of P.G. Studies and Research in Biological Science, Rani Durgavati Vishwavidyalaya, Jabalpur (M.P.), India, which was previously isolated from flour mill soil sample situated in Station Sadar Canteen, Jabalpur, Madhya Pradesh, India. The strain was maintained through periodic transfer after every four weeks on Luria-Bertani (LB) agar slant (pH 7) and stored at 4°C.

Initial culture condition for asparaginase production

Asparaginase production was carried out in modified basal semi synthetic broth medium composed of: 3g glucose; 6.0g Na₂HPO₄·2H₂O; 1.77g KH₂PO₄; 0.5g NaCl; 0.37g MgSO₄·7H₂O; 0.015g CaCl₂·2H₂O; 5g asparagine; 1g yeast extract and 1g peptone with an initial pH of 7.0 (Kumar *et al.*, 2011). The loop full culture was inoculated in Erlenmeyer flasks and was further incubated at 37°C in 180 rpm for 12 h. From this culture, 2% inoculum (v/v) (A₆₀₀ = 0.6-0.8) was further transferred to 50 ml aforementioned fresh sterile medium and further incubated at 37°C in 180 rpm for 24 h. The culture was centrifugation at 10,000 rpm for 5 min to obtained crude extract. Cell free supernatant was served as source of enzyme to determine asparaginase activity. Bacterial biomass was determined by measuring the absorbance at 600 nm (Henroette *et al.*, 1993). All the experiments were carried out in triplicates.

Assay for asparaginase

Asparaginase activity was calculated as we described previously (Sharma *et al.*, 2014). One international unit of asparaginase (IU) is defined as the amount of enzyme that liberates 1 μmol of ammonia min^{-1} at 37°C.

The total protein contents were determined by following the method of Lowry *et al.*, 1951, using bovine serum albumin (BSA) as standard.

Optimization of medium for asparaginase production

The various physiological and nutritional factors were evaluated for maximum production of asparaginase using one factor at a time approach from *O. intermedium*.

The optimized parameter obtained was incorporated and followed till final composition and conditions were obtained. The different parameters includes were batch time, pH, temperature, inoculum density, carbon, nitrogen, mineral ions and amino acids for maximum enzyme production.

Inoculum preparation and batch time

The 24h old pure culture of bacterium *O. intermedium* was transferred in 20 ml of aforementioned sterile medium and flask was incubated overnight at 37°C in a rotary shaking incubator (Remi C-24 BL) at 180 rpm. The 2% (v/v) inoculum ($A_{600}=0.6-0.8$) of this culture was transferred in 100 ml sterile basal semi synthetic medium grown in 250 ml Erlenmeyer flasks and incubated at 37°C with shaking at 180 rpm for 12 h.

The 5 ml medium was withdraw at regular interval of 3h and centrifuged at 10,000 rpm. The supernatant was used for Asparaginase estimation.

Effect of size of inoculum

To determine influence of size (%) of inoculum on asparaginase production, different sizes (0.5- 5% v/v) was used to inoculate in 250 ml Erlenmeyer flask containing 50 ml basal semi synthetic medium. The medium with inoculum size 2% v/v was set as control. Flasks were incubated at optimized incubation period at 37°C with shaking at 180 rpm. Cell growth and asparaginase activity was determined.

Effect of pH

The effect of pH on bacterial growth and asparaginase production was examined in the pH range 4.0-9.0.using 1N HCL and 1N NaOH as appropriate.

The medium with the pH -7 was set as control. Flasks were incubated at optimized incubation period at 37°C with shaking at 180 rpm. Cell growth and and asparaginase activity was determined.

Effect of temperature

Different temperatures (i.e. 25, 30, 35, 37, 40, 45, 50°C) were evaluated for their effect in asparaginase production by growing the bacterium in the above optimized medium. The medium with temperature 37°C was set as a control. Cell growth and asparaginase activity were estimated.

Effect of carbon

The effect of carbon source on production of the enzyme various carbon sources were tested (0.3%) i.e. sucrose, maltose, mannose, galactose, lactose, glycerol, fructose, starch and sodium acetate by substituting glucose from the medium and with negative control. Cell growth and Asparaginase activity was determined.

Effect of nitrogen

The various nitrogen sources (0.5%) like beef extract, yeast extract, casein, soya flour, tryptone, urea, ammonium oxalate, ammonium chloride, ammonium nitrate, ammonium sulphate were examined for their effect on Asparaginase production by substituting peptone in the medium containing optimum carbon source. Cell growth and Asparaginase activity was determined by standard asparaginase assay.

Effect of mineral ions

The effect of various ions sources (0.3%) viz. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, KH_2PO_4 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl , Na_2HPO_4 were examined for their effect on L- asparaginase production by substituting NaCl in the medium. Cell growth and asparaginase activity was determined.

Effect of amino acids

The effect of various amino acids (0.3%) viz. L-histidine, L-ornithine, L-asparagine, L-arginine, L-glutamine, L-aspartic acid and negative control by substituting asparagine in the medium Flasks were inoculated and incubated at aforementioned optimized conditions. Cell growth and asparaginase activity was determined.

Results and Discussion

Effect of batch time

The effect of batch time on asparaginase production, culture flask was incubated at 37°C and 180 rpm. After 3 h, 5mL culture was withdrawn, and cell free supernatant was evaluated for asparaginase activity.

The maximum level of enzyme production $2.788 \pm 0.07 \text{ IU mL}^{-1}$ along with cell growth

($A_{600} = 2.242 \pm 0.09$) was obtained in batch time of 18h (Table 1). Although, extended incubation period led to the low yield of asparaginase activity may be due to rapid depletion of nutrients in the medium.

Effect of size of inoculum

The inoculum size is known to affect the growth of an organism and thus plays important role in enzyme production. The effect of different densities 0.5- 5% v/v were examined (Table 2).

The maximum asparaginase yield was obtained in inoculum size of 2% (v/v) ($3.789 \pm 0.02 \text{ IU mL}^{-1}$) whereas growth of bacteria was found maximum in 3.5% inoculum size.

Any further increase or decrease in inoculum density led to reduction in asparaginase production. Therefore, 2% inoculum size was selected for further medium optimization studies.

Effect of pH

In order to find pH for maximum asparaginase production, the strain *O. intermedium* was grown in different pH range from 5.0-9.0. The asparaginase produces in the range of (5.0-8.5) (Table 3). However, optimum pH for maximum yield was found $3.998 \pm 0.06 \text{ IU mL}^{-1}$ along with growth of the bacteria $A_{600} = 2.443 \pm 0.05$ in pH-7 (Control). Further increase in pH causes decrease in both the growth and enzyme production. Therefore, pH 7.0 was selected for further media optimization studies.

Effect of temperature

The strain *O. intermedium* grew well in range of $25\text{-}50^\circ\text{C}$ (Table 4). The asparaginase yield was obtained maximum in the 40°C which is

5.856±0.05 IU mL⁻¹ along with optimal growth (2.353±0.04). After 40°C the enzyme production was declined. Therefore, temperature selected for further experiments was 40°C.

Effect of carbon source

The impact of selected carbon source was suitable and supported asparaginase synthesis (Figure 1). Wherein lactose was found to have maximum yield of asparaginase 8.606±0.05 IU mL⁻¹ along with growth A₆₀₀= 2.030±0.04 as compared to the glucose (control) where only 5.772±0.04 IU mL⁻¹ asparaginase was produced. Therefore, further studies were conducted using glycerol instead of glucose in the production medium.

Effect of nitrogen source

The effect organic and inorganic different nitrogen sources were used to evaluate their ability for higher yield of asparaginase production (Figure 2). The results obtained revealed that supplementation of yeast extract in the medium as nitrogen source resulted in

maximum yield of asparaginase 10.042±0.1 IU mL⁻¹ along with bacterial growth A₆₀₀ = 2.274 ± 0.03. Therefore, further optimization studies were conducted using yeast extract in the medium.

Effect of mineral ions

Among several tested mineral ion sources were incorporated in medium individually to determine their effect on asparaginase production (Figure 3). Results revealed that maximum enzyme yield 11.241±0.04 IUmL⁻¹ was achieved with MgSO₄.H₂O along with the growth of the organism. A₆₀₀=2.248±0.07 compared to other mineral ions.

Effect of amino acids

The effect of selected amino acids on asparaginase production by *O. intermedium* is summarized in (Figure 4). The maximal asparaginase yield 13.956± 0.07 IU mL⁻¹ was obtained by incorporation of asparagine along with the highest growth A₆₀₀=2.456±0.5 compared to other amino acids.

Table.1 Effect of batch time on growth and asparaginase production from *O. intermedium* were inoculated in 100ml medium at 37°C and 180

Batch time (h)	Absorbance (600 nm)	Asparaginase activity (IU/ml)
3	0.242±0.01	0.012±0.05
6	0.654±0.2	0.092±0.08
9	1.242±0.09	0.989±0.12
12	1.646±0.03	1.855±0.01
15	1.854±0.02	2.564±0.05
18	2.242±0.09	2.788±0.07
21	2.098±0.06	2.654±0.04
24	1.333± 0.07	1.756±0.05
27	1.246±0.05	1.542±0.03
30	1.064±0.09	1.323±0.02
33	0.999±0.08	0.898±0.03
36	0.899±0.04	0.343±0.02

Values represent the mean ±SD of triplicate measurements.

Table.2 Effect of different inoculum densities on growth and asparaginase production from *O. intermedium* were inoculated in 50ml medium at 37°C and 180

Inoculum size (%)	Growth (600 nm)	Asparaginase activity (IU/ml)
0.5%	1.121±0.04	1.211±0.02
1%	1.444±0.03	1.851±0.08
1.5%	2.051±0.07	2.775±0.03
2%	2.654±0.04	3.789±0.02
2.5%	2.789±0.02	2.756±0.06
3%	2.981±0.1	2.211±0.08
3.5%	3.211±0.04	1.898±0.07
4%	3.01±0.06	1.754±0.012
4.5%	3.212±0.08	1.665±0.02
5%	3.222±0.04	1.654±0.03

Values represent the mean ±SD of triplicate measurements.

Table.3 Effect of pH on growth and asparaginase production from *O. intermedium* 2% inoculum was inoculated in 50ml medium of different pH at 37°C for 18 h in 180 rpm

pH	Growth (600 nm)	Asparaginase activity (IU/ml)
5.0	0.976±0.02	0.654±0.06
5.5	1.444±0.03	1.843±0.05
6.0	1.865±0.06	2.243±0.02
6.5	2.01±0.03	2.665±0.07
7.0	2.243±0.05	3.998±0.06
7.5	2.111±0.05	2.101±0.05
8.0	1.654±0.04	0.999±0.08
8.5	1.242±0.08	0.464±0.04
9.0	0.891±0.06	-

Values represent the mean ±SD of triplicate measurements.

Table.4 Effect of different temperature on growth and asparaginase production from *O. intermedium* with 2% inoculum in 50ml medium flask (pH 7.0) and Incubated for 18h at 180 rpm

Temperature (°C)	Growth (600 nm)	Asparaginase activity (IU/ml)
20	0.112±0.1	0.025±0.02
25	0.775±0.03	0.456±0.02
30	1.441±0.05	0.875±0.03
37	2.243±0.05	3.741±0.07
40	2.353±0.04	5.856±0.05
45	2.111±0.04	4.245±0.04
50	1.456±0.02	1.845±0.05

Values represent the mean ±SD of triplicate measurements.

Fig.1 Effect of different carbon sources on growth and asparaginase production from *O. intermedium* with 2% inoculum in 50ml medium flask (pH 7.0) and Incubated for 18 h at 40°C in 180 rpm

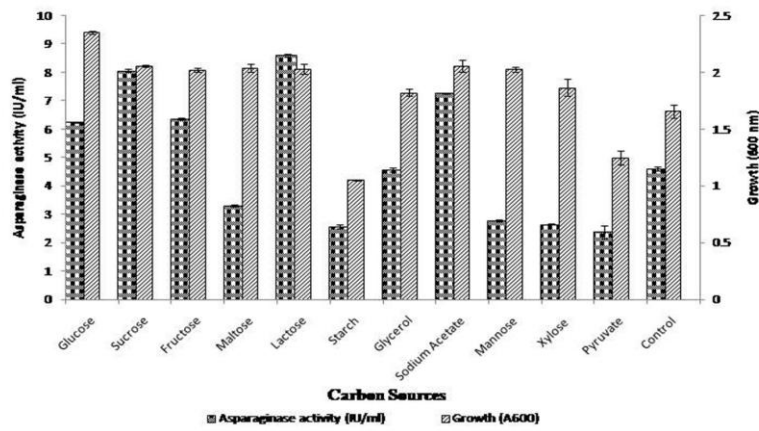


Fig.2 Effect of different nitrogen sources on growth and asparaginase production from *O. intermedium* with 2% inoculum in 50ml medium flask (pH 7.0) and Incubated for 18 h at 40°C in 180 rpm

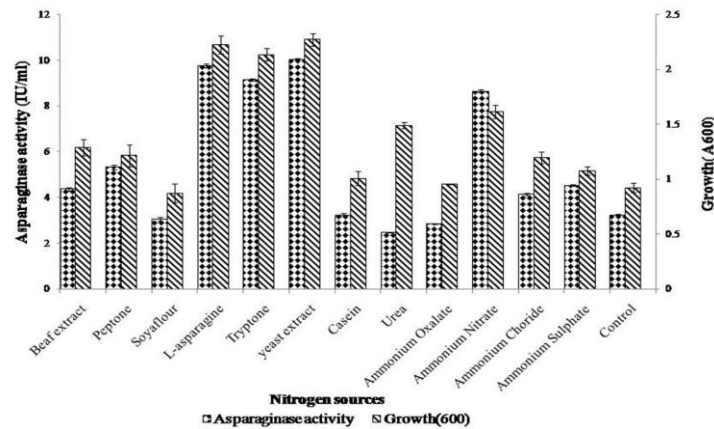


Fig.3 Effect of different mineral ions sources on growth and asparaginase production from *O. intermedium* with 2% inoculum in 50 ml medium flask (pH 7.0) and Incubated for 18h at 40°C in 180 rpm

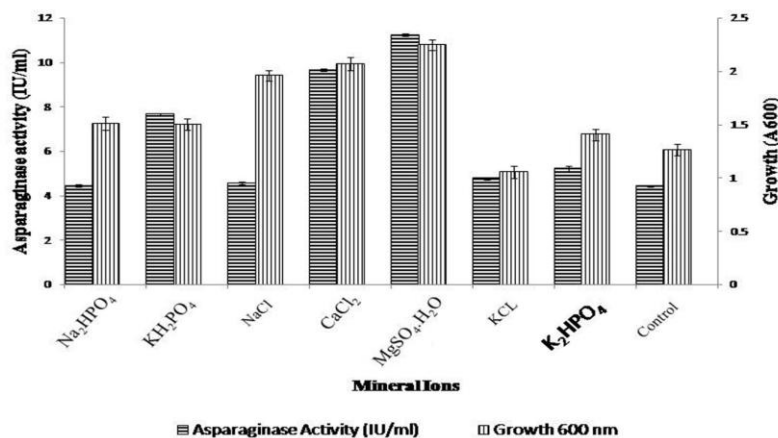
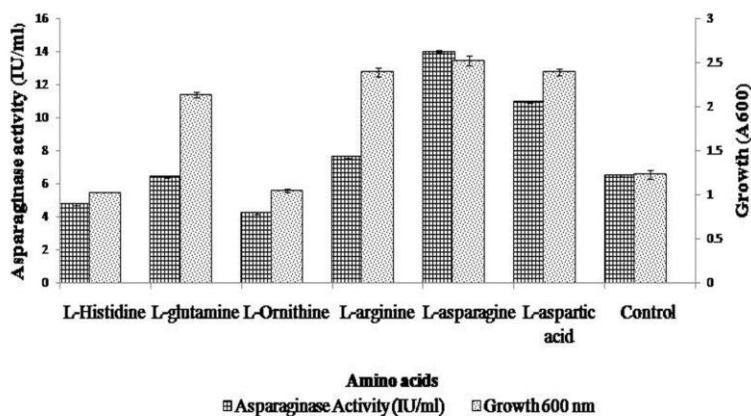


Fig.4 Effect of different amino acids sources on growth and asparaginase production from *O. intermedium* with 2% inoculum in 50 mL medium flask (pH 7.0) and Incubated for 18 h at 40°C in 180 rpm



Enzymes are highly selective catalytic proteins which regulate all biochemical processes of living organism. Asparaginase has been proved to be promising enzyme in pharmaceutical Indus try as well as in food industry for the production of acrylamide free food. Process optimization can give a structural approach to the identification of optimal condition for the maximum synthesis of asparaginase (Kenari *et al.*, 2011). Asparaginase produced from the bacterial strain *O. intermedium* isolated from the flour mill soil has potential in acrylamide inhibition was optimized for maximum enzyme production by one factor at a time approach and showed high specificity towards asparagine.

The batch time profile showed that maximum enzyme yield was achieved during stationary phase (18 h). This short batch time can be more significant for large scale production of enzyme. Our results are also comparable to Mahajan *et al.*, 2012 who also reported that the enzyme production was maximum in 18 h. Extended period of incubation might lead to the decomposition of enzyme because of interaction with other components in the media (Ramesh & Lonsane 1987).

The metabolic activities of the microorganisms are sensitive to the pH changes and the pH of the culture media has marked effect on the type and amount of enzyme produced. Each enzyme has specific pH optima for its activity. The yield of asparaginase was increased 1.445 fold in pH 7.0. This might be due to that *O. intermedium* grown rapidly at near neutral or the asparaginase might be more stable in this range of pH. Our studies are related with the previous finding of Geckil & Gencer (2004) they were reported that the production of asparaginase was down regulated linearly with pH of the medium, and the optimal activity was observed at neutral and alkaline pH. Abdel-Fattah & Olama, 2002 have also reported that the production of the enzyme from different microorganisms in modest alkaline range i.e from 7-8.

The temperature was found to influence bacterial extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane Rahman *et al.*, 2005. An increase in 2.117 fold enzyme activities was observed at 40°C and on exceeding the optimal temperature i.e. 45°C, the enzyme activity was dropped down likely due to heat dissipation which could lead to further drop in

the oxygen level and thereby reducing the growth and enzyme production. Our results are also comparable with Sharma *et al.*, 2015 who also reported the maximum enzyme production at the same temperature by *Enterobacter cloacae* isolated from rhizospheric soil of *Calendula officinalis*. Cell growth and the accumulation of metabolic products are strongly influenced by medium composition such as carbon, nitrogen, growth factors and inorganic salts (Thenmozhi *et al.*, 2011). The isolate was capable of utilizing a wide variety of carbon sources. Among carbon sources lactose was the best carbon source which enhanced 3.111 fold yield asparaginase production. In contrast to glucose, which was reported as a catabolism repression and catabolism inhibition of the components involved in lactate transport Garaev *et al.*, 1997 and lactate stimulated asparaginase synthesis. In the presence of glucose, low asparaginase yield were also reported in *Erwinia carotovora* Warankar & Khobragade 2010 and *Escherichia coli* Kenari *et al.*, 2011.

Maximum asparaginase production with 3.630 fold increase was obtained when yeast extract was used as sole organic nitrogen source for this strain. Organic sources were utilized more efficiently as compared to inorganic sources for enzyme production. Our studies also proportionate with Sharma *et al.*, (2015), they were obtained enhanced production of asparaginase by yeast extract as a nitrogen source.

The trace elements and metal ions are one of the required cofactors for enzyme production. Among various ion sources, the maximum asparaginase synthesis was found in the presence of $MgSO_4 \cdot H_2O$. This ion was indicated to have significant influence in asparaginase production (Kumar *et al.*, 2011; Kenari *et al.*, 2011). The presence of L-asparagine in the medium improved the

enzyme productivity by 5.045 fold with 13.956 ± 0.07 IUmL⁻¹ along with the growth $A_{600} = 2.456 \pm 0.05$ as compared to the control. This confirmed that *O. intermedium* was an inducible enzyme showed high specificity towards asparagine. However studied all other inducers were also found to improve enzyme level. Our studies are in the accordance with previous report of Singh & Shrivastava 2013 who reported 3 fold, increased of extracellular asparaginase synthesis from *Bacillus aryabhatai* ITBHU02 when medium supplemented with asparagine.

In conclusion, present study demonstrated that optimization of environmental and nutritional conditions for production of asparaginase from *O. intermedium* using one variable at a time approach. The biosynthesis of asparaginase from this strain was found maximum with batch time 18 h, inoculum age of 15h, inoculum size 2 %, initial pH 7.0 and temperature 40°C was used, along with lactose, yeast extract, $MgSO_4 \cdot H_2O$ and asparagine were best carbon, nitrogen, mineral ions, and inducer sources, respectively. The current study for the first time optimized the physical, nutritional parameters and enhanced 5.045 fold production of the asparaginase production successfully from *O. intermedium*. The study indicates that the production of the enzyme enhanced with higher yield in short time could be by optimized medium. The asparaginase production from *O. intermedium* could found to be a good alternative for further large scale production of the enzyme with its application in inhibition of acrylamide in starchy food after further studies and validation of the enzyme.

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