OPTIMIZATION OF L-ARGINASE PRODUCTION FROM *LACTOBACILLUS* SPECIES

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under the Supervision of

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WAKNAGHAT

TABLE OF CONTENTS

CERTIFICATE

This is to certify that project report entitled "***Optimization of L-arginase production from lactobacillus species*** ", submitted by ***Himanshi Gautam*** in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

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ACKNOWLEDGEMENT

Any assignment plus to litmus test of an individual's knowledge, credibility or experience and thus sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on my part to record thanks to them.

Therefore, on this was first of all I would like to thank my guide and mentor DR. SAURABH BANSAL for his guidance, help and constant encouragement throughout this project.

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I am also thankful to all my seniors and to the lab staff for their guidance and friendly cooperation.

Himanshi Gautam

Date:

List of Figures:-

List of tables:-

ABSTRACT

Arginases are anti-cancerous enzymes which can be used in the treatment of debilitating human cancers by nutritional starvation therapy. The antitumor activity of these enzymes is found to be effective in countering wide range of cancers.

One Variable at a time (OVAT) technique was employed to optimize the production of arginase from *Lactobacillus acidophilus*. Various physical parameters such as volume, inoculum age, inoculum size and incubation time were studied for their effect on the production of arginase and so on its activity and hence were optimized. The location of the enzyme was intracellular and the optimal values of physical parameters for arginase production were: volume = 200ml, inoculum age = 10 hours, inoculum size = 1% and incubation time = 24 hours.

OBJECTIVE

Optimization of physical parameters for enhancing the L-arginase production

- Volume
- Inoculum age
- Inoculum size
- Incubation time

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Arginase is an anti-cancerous enzyme which can help in treating cancer therefore it is a very high value enzyme. Nutritional starvation of cancer cells have been tested by many researchers and have been found to be successful in few cases. Cancer cells have utter requirements for nutrition, a few of its nutrients it can synthesize and for a few others like arginine and methionine it depends on the host cells (Hoshiya *et al*., 1995). If the cancer cells are deprived of these amino acids, they starve to death, since they can't synthesize these amino acids (Lishko *et al*., 1993).

At industrial level the medium composition and the process conditions play a critical role in enzyme production as they affect the formation, concentration and yield of a particular end product thus affecting the overall process economics, therefore it is important to consider the optimization of medium and process conditions in order to maximize the profits (Huang *et al.*, 2007).

So therefore, process optimization is aimed at maintaining optimum and homogenous reaction conditions minimizing microbial stress exposure and enhancing metabolic accuracy.

The cost of an enzyme is one of the main factors determining the economics of a process. The goal of basic research for industrial applications is to reduce the costs of enzyme production by optimizing the fermentation medium and process (Pal *et al*., 2010). In general, optimization by the traditional 'one-factor-at-a-time' technique is determined by varying one factor while keeping the other factors at a constant level. This method, although simple, often requires a considerable amount of work and time.

1.1.1 ***Arginase***

L-Arginase: Arginase is a 105 kDa homotrimeric enzyme which requires a metal ion like manganese for its catalytic activity (Kumar *et al*., 2012).Arginase is the terminal enzyme of the urea cycle among the six other enzymes. Arginases has the potential for regulating the availability of arginine for the synthesis of NO, polyamines, agmatine, proline and glutamate (Munder *et al*., 2009). Arginase was discovered in mammalian liver by Kossel & Dakin (1904) and found out that the products of its action are ornithine and urea. Since then, the enzyme has attracted various

research groups from many points of view. The enzyme has been found to exist in two forms that have evolved with differing tissue distributions, metabolic functions and subcellular locations in mammals (Kumar *et at*., 2012). The cytosolic form which is Arginase I is found predominantly in the liver or hepatic cells and is important in ureogenesis. Arginase II is a mitochondrial enzyme that is extra-hepatic (originating outside the liver) and more widely distributed in numerous tissues, for example, kidney, and skeletal muscle (Jenkinson *et al*., 1996). It may also be found at lower levels in macrophages, lactating mammary glands, and brain. Important roles of Arginase II have been reported which are biosynthesis of polyamines, the amino acid ornithine, proline and glutamate and in the inflammatory process. Genetic "knockout" experiments have been performed which suggests that arginase II functions in L- arginine homeostasis by regulating L-arginine concentrations for cellular biosynthetic reactions such as nitric oxide biosynthesis. The human type I and type II arginases are related by 58% sequence identity and are immunologically distinct.

1.1.2 ***Mechanism of action***

The arginases catalyse the divalent cation-dependent hydrolysis of L-arginine to form the non-protein amino acid L-ornithine and urea 11.

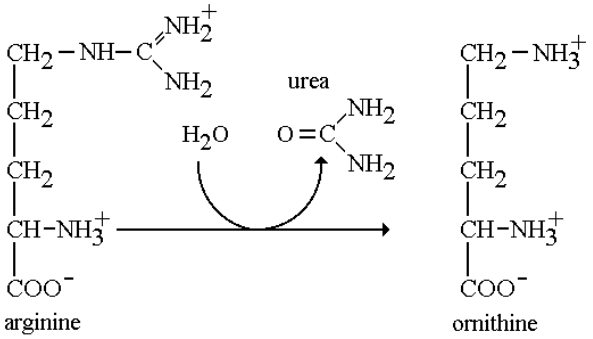

Figure 1: Reaction catalysed by arginase

In the organisms containing urease, urea is further converted to ammonia and carbon-dioxide.

Urea -------urease----------> $NH_4^+ + CO_2$

1.1.3 ***Occurrence and distribution*:**

Sources of L-arginase : Arginase apart from being ubiquitously present in mammalian tissues has also been characterized from various worms, molluscs, fishes, bacteria, fungi, yeast, actinomycetes, algae and plants.

Bacterial sources reported

Among bacteria producing Arginase, the prominent ones include:

- Mycobacteria (Zellar *et al*,. 1945)
- Proteus spp (Prozesky *et al*,. 1973)
- T.aquaticus (Degryse *et al*,. 1976)
- Agrobacterium-Rhizobium group (Dessaux *et al*,. 1976)
- Cyanobacterium aphanocapsa (Weathers *et al* ., 1978)
- Cyanobacteria (Gupta *et al*,. 1981)
- *Bacillus licheniformis* (Gupta *et al*,. 1981)
- *Bacillus subtilis* 168 (Simon *et al*,. 1976)
- *Streptomyces* spp. (Baumberg *et al*,. 1979)
- *Streptomyces calvuligerus* (Vargha *et al*,. 1983)
- *Streptomyces calvuligerus* (Bascaran *et al*,. 1989)
- Cyanobacterium *Anabaena cycadeae* (Singh *et al*,. 1994)
- Thermophile *Bacillus caldovelox* (Patchett *et al*,. 1991)
- *Rhodobacter capsulatus* E1 F1 (Moreno-Vivian *et al* ,. 1992)
- *Bacillus anthracis* (Raines *et al*,. 2006)
- *Chlamydia pneumoniae* (Hartenbach *et al*,. 2007)

1.1.4 *Arginase: The medical therapeutic*

The enzyme has been found to possess profound therapeutic benefits in treatment of various physiological disorders in the body.

- Measurement of circulating Arginase I i.e., serum arginase levels have been found to be used as rapid marker for liver injury (Puri and Kaul, 1995).
- Arginase has been found to be essential for the treatment of acute neurological disorders (Esch *et al.,* 1998).
- Ornithine, produced by arginase is necessary for the production of collagen, which has been found helpful in therapy of rheumatoid arthritis (Corraliza *et al.,* 2002).
- Arginase up regulates the synthesis of polyamines and proline via arginine hydrolysis which are necessary compounds for cell proliferation and growth (Mori and Gotoh, 2004).

- Arginase competes with NOS (Nitric oxide synthase) for endogenous arginine pools, Its levels acts as an indirect regulator of penile and vaginal flow thus they play an important role in male and female sexual arousal (Kim *et al.,* 2004).
- Up-regulation of endogenous arginase I causes the activation of some neural regeneration pathways, (Lange *et al.,* 2004) as the reaction is being facilitated by polyamines.
- It has been reported that arginase-I is constitutively expressed in human granulocytes and participates in fungicidal activity by a novel antimicrobial effector pathway likely through arginine deprivation in phagolysosome (Munder *et al.,* 2005).
- A deficiency of the liver enzyme results in hyperargininemia, an autosomal recessive disorder (Scaglia and Lee, 2006). Raised arginase activity in serum of 85% patients suffering from colorectal cancer liver metastases (CRCLM) led to the conclusion that Arginase can be a useful marker for diagnosis of CRCLM (Meilczarek *et al.,* 2005).
- Increase in psychological stress results in raising NO level which results in poor sperm quality. Thus, arginase levels are important and helpful to keep NO levels (via arginine degradation) in control for maintenance of semen quality (Eskiocak *et al.,* 2006).
- Arginase has been shown to be effective for treatment of Hepatitis- B (Cheng *et al*., 2006).
- Arginase I & II levels rise remarkably in asthma and lung infection thus, showing the involvement of arginase in the pathology of the disease (King *et al.,* 2003).
- Measurements of plasma arginase activity provide a useful marker for underlying metabolic disorder and efficacy of treatment for asthma (Morris *et al.,* 2004).

1.2 BACTERIA DESCRIPTION

Lactobacilli are reported to show diverse behaviour and hence have attracted attention as a potential source of enzyme production (Kim *et al*., 1983).

Lactobacillus acidophilus (New Latin 'acid-loving milk-bacterium') is a gram positive, rod shaped coccobacilli, occurring singly or in chains. *L. acidophilus* is a homofermentative, microaerophilic species, fermenting sugars into lactic acid, and grows readily at rather low pH values (below pH 5.0) and has an optimum growth temperature of around 37 °C (99 °F) (Baati *et al.,* 2000).

The bacterial strain shows remarkable growth at pH between 5 and 6 (Pyar *et al.,* 2014).

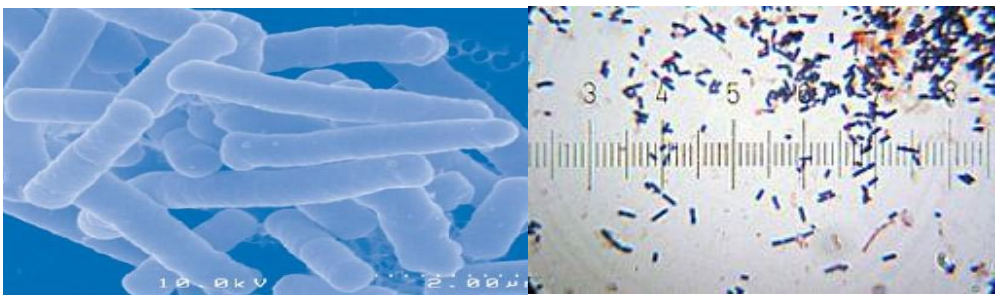

Figure 2: Gram staining of *Lactobacillus acidophilus* (Hansen *et al.*, 1970)

L. acidophilus occurs naturally in the human and animal gastrointestinal tract and mouth. Some strains of L. acidophilus may be considered to have probiotic characteristics. These strains are commercially used in many dairy products (Ljungh *et al.*, 2006).

The genus Lactobacillus is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economic importance. Strains of *L. acidophilus* were introduced into dairy products because of the potential advantage of consuming active LAB adapted to the intestine and to produce mildly acidified yoghurts (Schillinger *et al.*, 1999).

1.3 OPTIMIZATION TECHNIQUE

Optimization is a process by which one can get best combination of the parameters which can give us the best response with reduced costs and can help to maximize the profits from fermentation process.

A reaction can be thought of as a system accepting a number of inputs (parameters) and providing one or more outputs. Examples for inputs might include: temperature; solvent; pH; catalyst; and time. Examples for outputs might include: yield; selectivity; purity; and cost. The goal of reaction optimization is to select the best inputs to achieve a given output.

A popular way to deal with the multi-variable nature of reaction optimization is the One Variable at a Time (OVAT) approach. Here, all experimental inputs, except one, are kept constant, let's say time. An output, let's say yield, is then recorded at multiple time values. In this way, the "optimal" reaction time is revealed. This reaction time is then kept constant and another variable is chosen, for example temperature. The process continues until all inputs have been probed and a set of optimal inputs have been determined.

1.4 ENZYME ACTIVITY AND SPECIFIC ACTIVITY

The **enzyme** unit (U) is a unit for the amount of a particular **enzyme**. One U is **defined** as the amount of the **enzyme** that produces a certain amount of **enzymatic activity**, that is, the amount that catalyses the conversion of 1 micro mole of substrate per minute.

Enzyme activity: Ornithine released / Vol. of enzyme × Incubation time

Units= µM/ml/min

This is the activity of an enzyme per milligram of total protein (expressed in µM $min^{-1}mg^{-1}$). Specific activity gives a measurement of enzyme purity in the mixture. It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins. Specific activity is equal to the rate of reaction multiplied by the volume of reaction divided by the mass of total protein.

Specific activity: Enzyme activity / Total protein

Units= µM/ml/min/mg

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ***Microorganism***

Lactobacillus acidophilus was used for production of arginase.

Strain was procured from National dairy research Institute, Karnal.

2.1.2 ***Medium composition for maintenance of culture***

Lactobacillus acidophilus was revived and maintained in MRS broth.

Composition g/l

Enzymatic Digest of Animal Tissue	10 g
Beef Extract	10 g
Yeast Extract	5 g
Dextrose	20 g
Sodium Acetate	5 g
Polysorbate 80	1 g
Potassium Phosphate	2 g
Ammonium Citrate	2 g
Magnesium Sulphate	0.1 g
Manganese Sulphate	0.05 g

Final pH: 6.5 ± 0.2 at 37°C

2.1.3 ***Instruments used***

- Digital pH meter
- UV-Visible spectrophotometer

- Incubator shaker
- Centrifuge
- Autoclave
- Laminar Air Flow Cabinet
- Water bath
- Sonicator

2.1.4 ***Composition of minimal media used for production of arginase***

Constituents	Amount
KH_2PO_4	13.6g
$(NH_4)_2SO_4$	2g
$CaCl_2.2H_20$	0.01g
$MgSO_4.7H_2O$	0.2g
$FeSO_4.7H_2O$	0.5mg
Yeast extract	0.3g
Arginine	10mM

2.1.5 ***Conditions for production of arginase***

- ✓ pH= 5.0 – 5.5
- ✓ Temperature= 37
- ✓ Agitation speed=200 rpm
- ✓ Time= 8 hours

2.2 METHODOLOGY

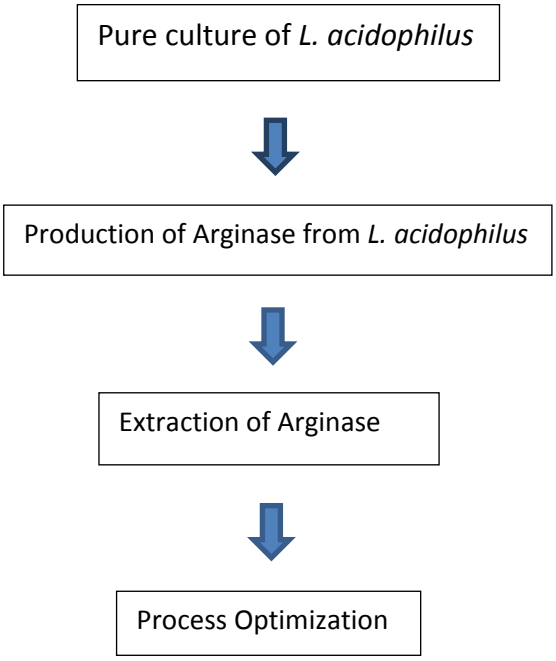

Figure 3: Methodology for L-arginase production optimization

2.2.1 ***Standard curves***

A standard curve (OD vs. concentration) was plotted by taking ornithine which is the product of different concentrations. This curve helps in determining the amount of ornithine produced.

One more standard curve was plotted by taking BSA (Bovine serum albumin). This curve helps in determining the amount of protein produced by the bacteria.

2.2.2 ***Production and extraction of enzyme***

2.2.2.1 Location of enzyme

Lactobacillus acidophilus was revived in MRS broth and then sub-cultured into minimal media for inoculum preparation. Then the media was prepared and inoculated with 2ml of the prepared inoculum. Then the shake flask was incubated and agitated for 8 hours to get enough growth of the bacteria. The enzyme activity was then checked for both supernatant and the pellet which was obtained by centrifugation and the cells were sonicated using lysis buffer and sonicator to break open and release the enzyme if produced intra-cellularly.

2.2.2.2 Selection of lysis buffer

One experiment was carried out to choose the best lysis buffer which would do the best cell lysis.

Different combinations of components were designed and checked to choose for the best effective cell lysis.

NaCl solution was also checked for lysis. (6 mg/ml)

Different compositions of lysis buffer (15 mM for 15 ml)

1) Lysis buffer 1	*2) Lysis buffer 2*	*3) Lysis buffer 3*
50 mM Tris (pH 8.0)	50 mM Tris (pH 8.0)	50 mM Tris (pH 8.0)
10% Glycerol	10% Glycerol	10% Glycerol
0.1% Triton – X100	0.1% Triton -X100	100 µg/ml Lysozyme
100 µg/ml Lysozyme		

2.3 ARGINASE ACTIVITY ASSAY

The colorimetric arginase assay measures the amount of ornithine by the appearance of an orange colour (read spectrophotometrically at 515 nm) from the reaction of ornithine with ninhydrin at low pH.

Procedure:

- **Heat activation step**: Equal volumes of extract and 10 mM $MnSO_4$ were pre-incubated for 30 min at 50 ºC to activate the enzyme.
- Next, arginase buffer [15 mM Tris, pH 7.5 plus 10 mM L-arginine was added and incubation continued at 37ºC.
- After 1 h the reaction was stopped by the addition of 750 µl of acetic acid, and the colour developed by the addition of 250 µl of ninhydrin (4 mg/ml)1) at 95 ºC for 1 h.

	Test (µl)	Control (E+B) (µl)	Control (S+B) (µl)	Blank (µl)
Metal ion	25	25	25	25
Crude enzyme	25	25	-	-
Substrate	50	-	50	-
Buffer	150	200	175	225
Glacial acetic acid	750	750	750	750
Ninhydrin	250	250	250	250

Table1: Volume of components be added for enzyme assay

(E=Enzyme, B=Buffer & S= Substrate)

2.4 LOWRY METHOD

Lowry method is used to determine the protein concentration in the solution. The total protein concentration is exhibited by a colour change of the sample solution in proportion to protein concentration which can be measured by absorbance at 750 nm.

Procedure:

- 100 µl of sample was taken in test tubes and then 900 µl distilled water was added to this to make the final volume 1 ml. 1 ml distilled water was taken as blank.
- 5 ml of Lowry's reagent was added in the sample containing test tubes and was incubated for 10 minutes at room temperature.
- After incubation 0.5 ml of Folin reagent was added and was then incubated for 30 minutes in dark.
- The absorbance at 750 nm was taken of each sample.

2.5 OPTIMIZATION OF PROCESS PARAMETERS FOR THE PRODUCTION OF L- ARGINASE AT SHAKE FLASK LEVEL

The various physical parameters were studied and optimized for the production of L- arginase at shake flask level.

2.5.1 ***Preparation of the starter culture***

The strain was grown in de Man, Rogosa and Sharpe broth (MRS) and then sub-cultured to minimal media and grown at 37ºC for 10 hours at 200 rpm.

2.5.2 ***Optimization of volume for L-arginase production***

To study the effect of volume on L- arginase production different volumes of media and ranging from 10 ml, 50 ml, 100 ml, 200 ml and 500 ml was prepared, inoculated (2% of inoculum) and incubated. Following growth of micro-organism, enzyme was extracted, assayed and the best optimum volume was chosen.

2.5.3 ***Optimization of inoculum age***

To study the effect of inoculum age on arginase production, flasks were incubated with inoculum having inoculum ages of 4hrs, 6 hrs, 8 hrs, 10 hrs and 12 hrs. Optimum inoculum age for further culturing was selected by means of assay of enzyme produced in various flasks inoculated with inoculum of different ages.

2.5.4 ***Optimization of inoculum size for L-arginase production***

To study the effect of inoculum size on arginase production, flasks ware incubated with inoculum sized ranging from 0.5%, 1%, 2%, 4% and 5% inoculum. Following the growth of micro- organism the enzyme was extracted, assayed and best optimum inoculum size was chosen.

2.5.5 ***Optimization of incubation time***

To study the effect of incubation time on arginase production, flasks were incubated for different times ranging from 4 hrs, 8 hrs, 16 hrs and 24 hrs. Optimum incubation time was selected by means of assay of enzyme produced.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 STANDARD CURVES

3.1.1 *Ornithine standard*

A standard curve was made for the quantification of the ornithine produced as shown in the figure below.

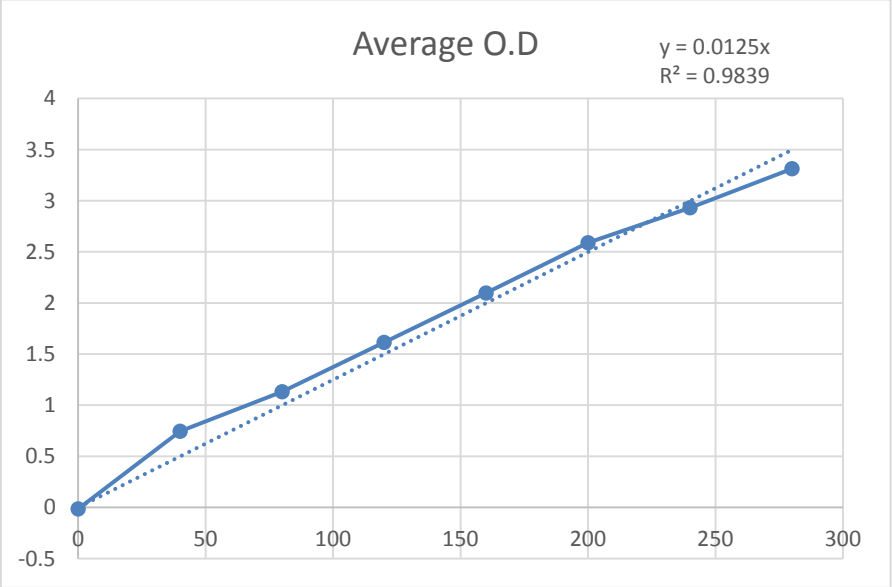

Figure 4: Standard Graph for Ornithine produced.

Ornithine (µg/ml)	Average O.D
0	0
40	0.74
80	1.13
120	1.61
160	2.1
200	2.6
240	2.93
280	3.31

Table 2: Ornithine concentration and its absorbance at 515nm

3.1.2 ***Bovine serum albumin (BSA) Standard***

A standard was made for the quantification of the protein concentration in the solution as shown in the figure.

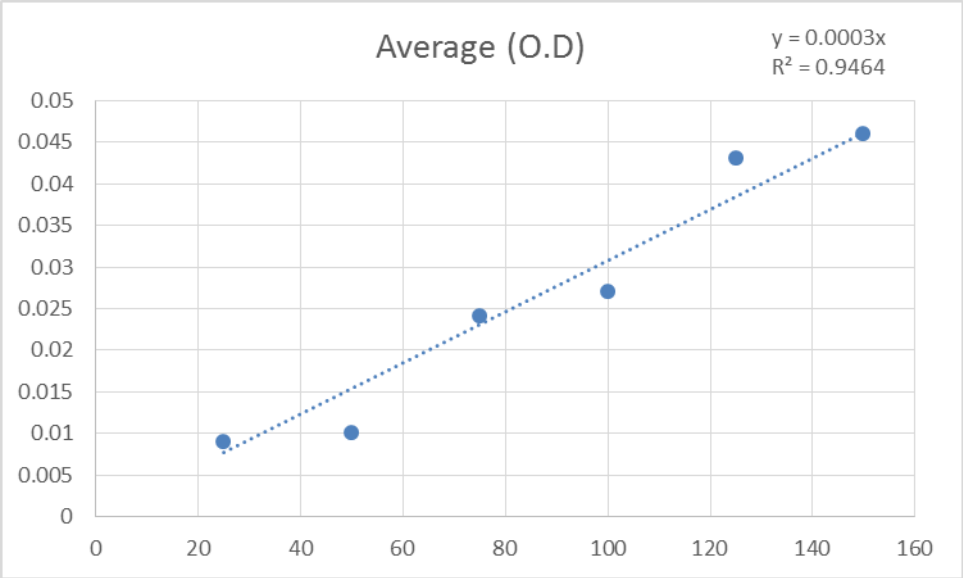

Figure 5: Standard Graph for protein produced.

BSA (µg/ml)	Average (O.D)
25	0.009
50	0.01
75	0.024
100	0.027
125	0.043
150	0.046

Table 3: BSA concentration and its absorbance at 750nm

3.2 LOCATION OF ENZYME

Enzyme assay was performed for both the supernatant and the pellet and the enzyme was found to be intracellular as the supernatant showed no activity.

Activity of the pellet was measured and it was found to be 1.09µM ml^{-1} min^{-1}

3.3 SELECTION OF LYSIS BUFFER

Enzyme assay was performed of arginase extracted using different lysis buffer and NaCl solution . Maximum activity was observed with lysis buffer 3 of composition: 50mM Tris (pH 8.0) ,10% Glycerol and 100µg/ml Lysozyme

Sample	Ornithine (µM/mL)	Unit activity ($\mu M\ ml^{-1}\ min^{-1}$)	Specific activity ($\mu M\ ml^{-1}\ min^{-1}$)
NaCl	0.081	0.05	1.15
LB1	0.079	0.05	0.09
LB2	0.06	0.04	0.1
LB3	0.16	0.11	0.2

Table 4: Unit activity and Specific activity at varying lysis buffers.

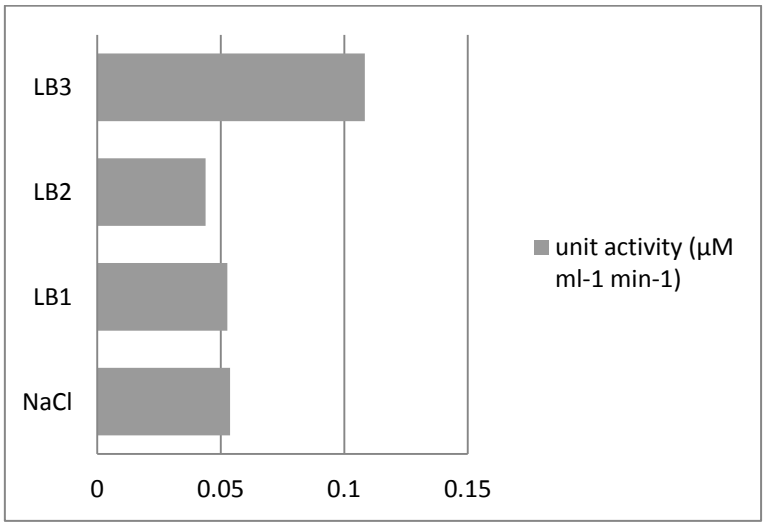

Figure 6: Unit activity with different lysis buffers

3.4 OPTIMIZATION OF PROCESS PARAMETERS

Process optimization was successfully completed and results were obtained in the forms of graphs. Parameters like,

- ✓ Volume

- ✓ Inoculum age
- ✓ Inoculum size
- ✓ Incubation time

Which are required for the growth of *Lactobacillus acidophilus* and arginase production were optimized.

3.4.1 ***Volume Optimization***

Arginase activity and specific activity was measured at different volumes and it was found that at 200ml specific activity was maximum that is 2.54 µM ml^{-1} min^{-1}

Volume (ml)	Ornithine released (µM/mL)	Unit activity (µM ml^{-1} min^{-1})	Specific activity (µM ml^{-1} min^{-1})
10	0.28	0.19	0.81
50	0.18	0.59	2.43
100	0.06	0.73	2.48
200	0.06	0.75	2.54
500	0.06	0.82	0.24

Table 7: Unit activity and specific activity at varying volumes

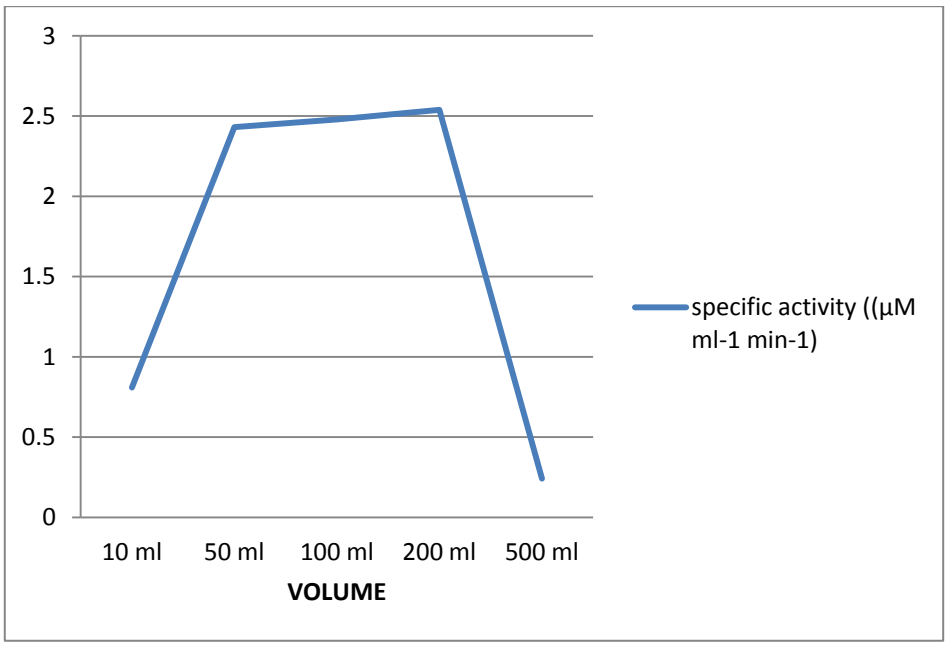

Figure 7: Specific activity at varying volumes.

3.4.2 *Inoculum Age optimization*

Enzyme Activity and specific activity was measured of enzyme produced by using inoculum of varying inoculum ages of 4hrs, 6hrs, 8hrs, 10hrs and 12hrs.

Maximum specific activity was obtained in enzyme produced with inoculum of age 10 hours that is 0.67 µM $ml^{-1}min^{-1}$.

Inoculum age (hr)	Ornithine (µM/mL)	Unit activity (µM ml^{-1} min^{-1})	Specific activity (µM ml^{-1} min^{-1})
4	0.028	0.37	0.25
6	0.065	0.87	0.6
8	0.053	0.71	0.48
10	0.079	1.05	0.67
12	0.084	1.12	0.47

Table 8: Unit activity and specific activity at varying inoculum age

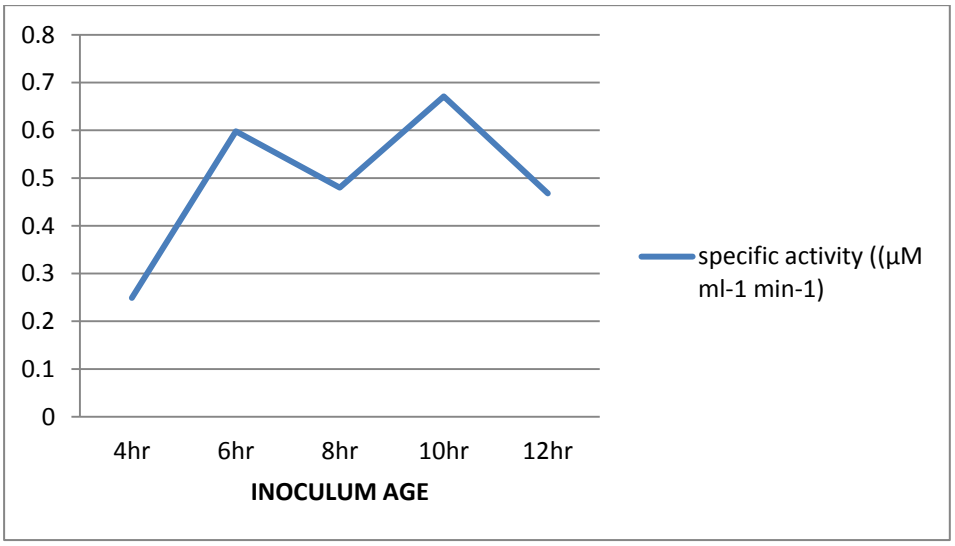

Figure 8: Specific activity at varying inoculum ages.

3.4.3 ***Inoculum size optimization***

Enzyme activity and specific activity was measured after inoculating with increasing amount of culture and it was found that with 1% inoculum size the specific activity was maximum.

Inoculum size	Ornithine released (µM/ml)	Unit activity (µM ml^{-1} min^{-1})	Specific activity (µM ml^{-1} min^{-1})
0.5%	0.059	0.78	7.81
1%	0.11	1.52	14.80
2%	0.11	1.47	14.71
4%	0.09	1.18	7.69
5%	0.07	0.96	7.99

Table 9: Unit activity and specific activity at varying inoculum sizes

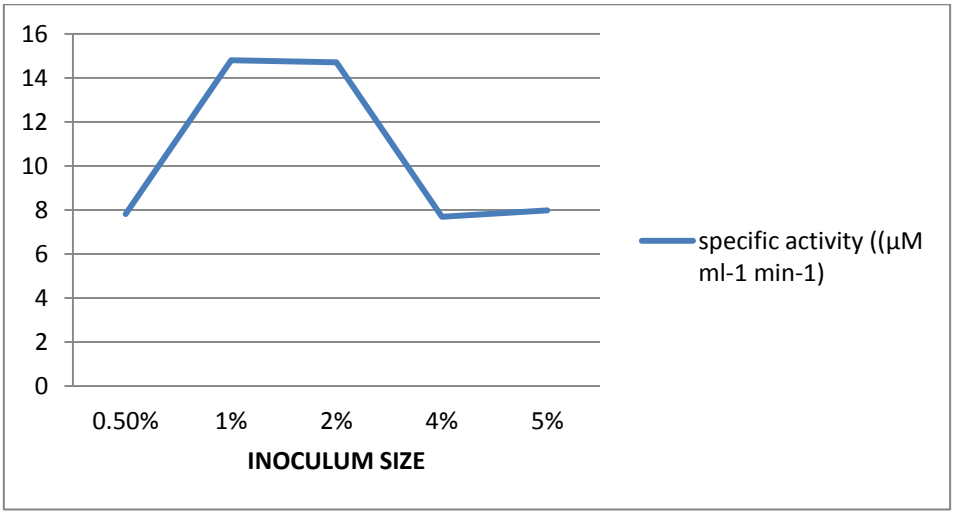

Figure 9: Specific activity at varying inoculum sizes.

3.4.4 ***Incubation time optimization***

Enzyme activity and specific activity was measured after incubating the inoculated flasks for varying times from 4 hours to 24 hours and the incubation time giving the maximum specific activity was selected.

Incubation time (hr)	Ornithine released (µM/ml)	Unit activity (µM ml^{-1} min^{-1})	Specific activity (µM ml^{-1} min^{-1})
4	0.06	0.88	2.04
8	0.04	0.58	1.24
16	0.07	0.99	2.07
24	0.07	0.99	2.10

Table 10: Unit activity and specific activity at varying incubation times

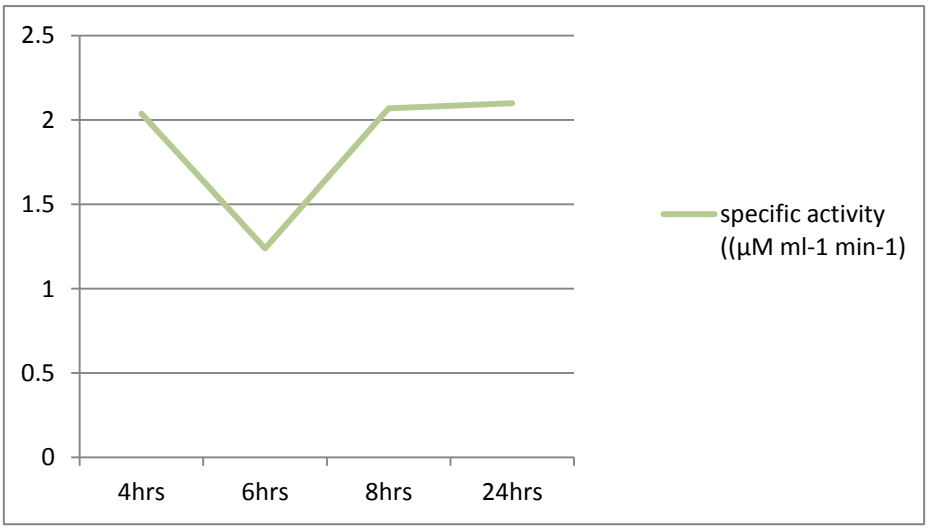

Figure 10: Specific activity at varying incubation times.

CHAPTER 4

CONCLUSION

Arginase is produced intracellularly by *Lactobacillus acidophilus* and can be extracted by suspending the cells in lysis buffer containing lysozyme and using a sonicator.

The optimum conditions to produce arginase were found out to be:

- Volume – 200ml
- Inoculum age – 10 hours
- Inoculum size- 1%
- Incubation time – 24 hours

From the results it was concluded that the unit activity increases with increase in the volume and the increase in protein content was also seen. Further moving to next parameter the inoculum age it was observed that unit activity increases with inoculum age. During optimization of inoculum age it was observed that the unit activity was increased with 2% and then a decrease in unit activity was seen.

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