Bioprocess Optimization for Scalable O-Antigen Production in Vaccine Development

Eswar Reddy K.[1,2]* and Venkateswarulu T.C.[1]
1. Department of Biotechnology, Vignan's Foundation for Science, Technology and Research, Vadlamudi – 522213, Andhra Pradesh, INDIA
2. Biological E Limited, Biotech Park, Phase II, Hyderabad, Telangana, INDIA
*ekavala@gmail.com

Abstract

O-polysaccharides are the main components in bacterial pathogenesis and vaccine formulations, making their large-scale production essential for polysaccharide and conjugate vaccine development. However, exact optimization of bacterial growth conditions, fermentation techniques and hydrolysis parameters is necessary to achieve high-yield and high-purity polysaccharide extraction. This study focuses on systematically enhancing O-polysaccharide production from Salmonella enterica serovar Paratyphi by refining key growth factor variables. The effects of temperature, pH, aeration, nutrient supplementation and glucose feeding on bacterial growth and polysaccharide biosynthesis were assessed using a thorough experimental design. Large-scale fed-batch fermentation yielded superior results over batch fermentation, ensuring sustained bacterial growth and continuous glucose availability for enhanced O-polysaccharide.

The acid hydrolysis process was optimized to prevent polysaccharide degradation while maintaining structural integrity, with a 2-hour hydrolysis duration at pH 5.0 and 95°C identified as the optimal condition. Size-exclusion chromatography (SEC) analysis confirmed that prolonged hydrolysis led to molecular fragmentation, reinforcing the need for controlled extraction conditions. This study presents optimized factors for bioprocess development combined with scalable and reproducible approach for maximizing O-polysaccharide production, aligning with industrial demands for efficient bioprocess development.

Keywords: O-polysaccharide, Salmonella enterica, polysaccharide vaccine, fermentation optimization, acid hydrolysis, fed-batch fermentation, bacterial bioprocessing.

Introduction

Typhoid remains a significant public health challenge, particularly in developing countries where inadequate sanitation and contaminated water sources facilitate its spread. The disease is caused by Salmonella enterica serovars Typhi and Paratyphi, with Salmonella typhi historically being the predominant pathogen. However, recent epidemiological studies indicate a notable rise in Salmonella paratyphi infections, which now account for nearly half of all typhoid cases in some endemic regions. This shift poses a growing concern, as Salmonella paratyphi infections are clinically indistinguishable from Salmonella typhi infections, yet they remain largely overlooked by current vaccination strategies[2,8,17].

There are licensed vaccines against Salmonella typhi, a live attenuated oral vaccine (Ty21a) and the Vi Capsular Polysaccharide (ViCPS) vaccine, but they do not offer protection against enteric fever caused by Salmonella paratyphi due to antigenic differences between the serovars. Salmonella paratyphi, in particular, lacks the Vi capsular antigen targeted by existing vaccines, leaving populations vulnerable to infection. Given the increasing prevalence of Salmonella paratyphi and the limitations of existing vaccines, there is an urgent need to develop a broad-spectrum vaccine that provides cross-protection against both Salmonella typhi and Salmonella paratyphi[3-5,12,14,16].

One promising target for such a vaccine is the O:2 polysaccharide, a serovar-specific component of the lipopolysaccharide (LPS) layer in Salmonella paratyphi. Lipopolysaccharides play a crucial role in bacterial virulence and immune system recognition, making them viable candidates for vaccine development. The O:2 antigen, in particular, is composed of a distinct trisaccharide backbone, rhamnose (Rha), mannose (Man) and galactose (Gal), with additional modifications that confer its serogroup specificity. This antigen has been recognized for its ability to elicit an immune response and serves as a potential vaccine candidate for broader typhoid prevention strategies[1,7,9,11,15].

Despite its immunogenic potential, large-scale production of O:2 polysaccharides present several challenges. Bacterial polysaccharide production is highly dependent on growth conditions and even minor fluctuations in culture parameters can significantly impact antigen yield and purity. Key factors such as temperature, pH, agitation speed, media composition and fermentation strategy influence bacterial metabolism and polysaccharide synthesis. Large-scale production demands a precisely controlled bioprocess to ensure consistent antigen quality and high yield, making process optimization as a critical aspect of vaccine development[6,10,13].

The present study focuses on optimizing key parameters influencing the production of O:2 polysaccharide at three critical stages: shake flask culture, fermentation and polysaccharide extraction. Shake flask studies allow for the preliminary assessment of factors such as temperature, pH

and agitation speed, providing insight into the fundamental growth characteristics of Salmonella paratyphi. Fermentation optimization explores the role of glucose feeding strategies, nutrient availability and dissolved oxygen levels, all of which directly affect bacterial biomass and antigen production. Finally, the polysaccharide extraction phase examines the impact of acid hydrolysis conditions, purification efficiency and pH adjustment, ensuring maximum antigen recovery with minimal degradation.

The goal is to develop an optimized, scalable bioprocess for high-purity O:2 antigen production with direct applications in vaccine formulation. By employing a structured approach to bioprocess optimization, this research aims to establish a foundation for cost-effective and industrially viable antigen production, contributing to the ongoing efforts to develop an improved vaccine against typhoid fever. Given the increasing burden of Salmonella Paratyphi infections and the rising threat of antibiotic resistance, optimizing antigen production represents a crucial step toward strengthening global typhoid prevention strategies.

Material and Methods

Bacterial Strain and Culture Conditions: The bacterial strain Salmonella enterica serovar paratyphi (MTCC 3220) was procured from the Microbial Type Culture Collection and Gene Bank (MTCC). The strain was revived on trypticase soy agar (TSA) plates and incubated at 35°C overnight to obtain fresh colonies. These colonies were then inoculated into a liquid medium containing glucose (2 g/L), tryptophan (0.2 g/L), cystine (0.2 g/L), NaCl (2.5 g/L), $MgSO_4$ (0.2 g/L), $(NH_4)H_2PO_4$ (1.0 g/L) and K_2HPO_4 (1.0 g/L) to support optimal bacterial growth. The cultures were incubated at 200 rpm for 48 hours and aliquots were stored in 10% glycerol at -70°C for future use.

Optimization of Growth Factors: To maximize O-polysaccharide production, growth conditions were optimized in three stages: shake flask studies, fermentation and polysaccharide extraction.

Shake Flask Studies: Shake flask experiments were conducted to determine the optimal growth conditions for Salmonella enterica serovar paratyphi. The following parameters were evaluated:

(i) Temperature Optimization: Cultures were incubated at 30°C, 35°C and 40°C to assess the impact of temperature on bacterial proliferation and glucose consumption.

(ii) pH Optimization: The medium pH was adjusted to 5.5, 6.5 and 7.5 using 1M NaOH or 1M H_2SO_4. Cultures were grown under these conditions and OD600 measurements were taken periodically.

(iii) Agitation Studies: Bacterial cultures were subjected to 50 rpm, 100 rpm, 150 rpm, 200 rpm, 250 rpm and 300 rpm to evaluate the effect of oxygen availability on cell growth.

(iv) Nutrient Supplementation: The effect of nitrogen sources was studied using yeast extract (0.3-1% w/v) and Hysoy (1-3% w/v) and their impact on OD600 values was recorded.

(v) Negative Control Studies: The necessity of specific growth factors was evaluated by growing cultures with and without tryptophan, $MgSO_4$ and nicotinic acid to determine their essentiality. Each shake flask contained 300 mL of media and growth was monitored at 2-hour intervals using a UV spectrophotometer (OD600). Experiments were performed in triplicate and average values were used for further analysis.

Fermentation Studies: Following shake flask optimization, large-scale fermentation was performed in an 80 L bioreactor containing 75 L of the optimized culture medium. The fermenter was sterilized at 121°C for 20 minutes, after which it was inoculated with 3.25 L of actively growing culture. Fermentation was monitored under controlled conditions to assess the impact of glucose feeding on bacterial growth and polysaccharide production.

(i) Glucose Feeding Strategy: A comparative study between batch and fed-batch fermentation was conducted. In batch fermentation, glucose was fully consumed in 16 hours limiting further growth. In fed-batch fermentation, glucose feeding was initiated when OD600 reached ~20 ± 5, ensuring a minimum concentration of 2 g/L in the medium. Glucose consumption profiles were analyzed to establish correlations between nutrient availability and cell density, helping to determine the efficiency of substrate utilization. Polysaccharide yield trends were evaluated based on hydrolysis duration and extraction efficiency, ensuring optimal recovery conditions while minimizing degradation. These analyses provided a comprehensive understanding of the factors influencing bacterial growth, metabolic activity and polysaccharide production, facilitating the identification of the most effective process parameters.

(ii) Dissolved Oxygen (DO) Regulation: The DO level was maintained at 30% by adjusting aeration and agitation speeds.

(iii) Sampling and Analysis: Culture samples were collected every 2 hours to monitor OD600, glucose consumption (measured using a YSI analyzer) and metabolite production.

Polysaccharide Extraction: The O-polysaccharide was extracted using an acid hydrolysis method where the fermentation broth was subjected to controlled conditions to ensure maximum yield.

Hydrolysis Conditions: The pH of the fermentation broth was lowered to 5.0 ± 0.2 using acetic acid and the culture was heated to 95°C ± 5°C. Hydrolysis was performed for 1

to 6 hours, with samples being collected at hourly intervals to determine the optimal extraction duration.

Quantification of Polysaccharide Yield: The polysaccharide yield was estimated using the phenol-sulfuric acid assay, a widely used method for carbohydrate quantification. In this assay, 50 µL of an 80% phenol solution was added to each sample, followed by the addition of 2 mL of concentrated H_2SO_4. The reaction mixture was incubated for 10 minutes at room temperature to allow for complete color development. Absorbance was then measured at 490 nm using a UV-visible spectrophotometer. The total sugar concentration was determined using a standard glucose calibration curve.

Statistical Analysis: All experiments were conducted in triplicate and the results were expressed as mean ± standard deviation.

Results

Shake flask studies:

Effect of Temperature: The bacterial culture was incubated at different temperatures (30°C, 35°C and 40°C) to determine the optimal temperature for growth and polysaccharide production. Similar growth was observed at 30°C and 35°C, while lower growth was recorded at 40°C. Glucose consumption was highest at 30°C, indicating enhanced polysaccharide production at this temperature (Figure 1).

Effect of pH: The results demonstrated that optimal growth occurred at pH 6.5 ± 0.3, while deviations from this pH led to a decline in bacterial proliferation (Figure 2).

Effect of Agitation: Maximum growth was observed at 200–250 rpm, while lower agitation resulted in reduced bacterial proliferation. No significant improvement was noted at higher agitation speeds (Figure 3).

Effect of Nutrient Supplementation: The presence of yeast extract (Figure 4a) and Hysoy ((Figure 4b) at optimal concentrations significantly enhanced bacterial growth. The availability of nitrogen and carbon sources plays a pivotal role in bacterial proliferation and polysaccharide biosynthesis. The study examined the impact of yeast extract (0.3–1.0% w/v) and Hysoy (1–3% w/v) supplementation on OD600 values. The results indicated that higher concentrations of yeast extract (1.0% w/v) led to improved bacterial growth, suggesting its role in providing essential amino acids, peptides and growth stimulants. Similarly, Hysoy at 2.0% w/v yielded optimal growth with diminishing returns at 3.0% w/v, possibly due to metabolic imbalances caused by excess nitrogen.

These findings align with studies demonstrating that optimal nitrogen availability enhances polysaccharide yield whereas excess nitrogen can redirect metabolic flux towards biomass accumulation rather than exopolysaccharide production. Therefore, the controlled supplementation of yeast extract and Hysoy at optimal concentrations ensures a balanced metabolic state conducive to O-polysaccharide synthesis.

(i) Effect of Tryptophan and $MgSO_4$: Growth was significantly inhibited in the absence of tryptophan and $MgSO_4$, indicating their essential role in bacterial proliferation.

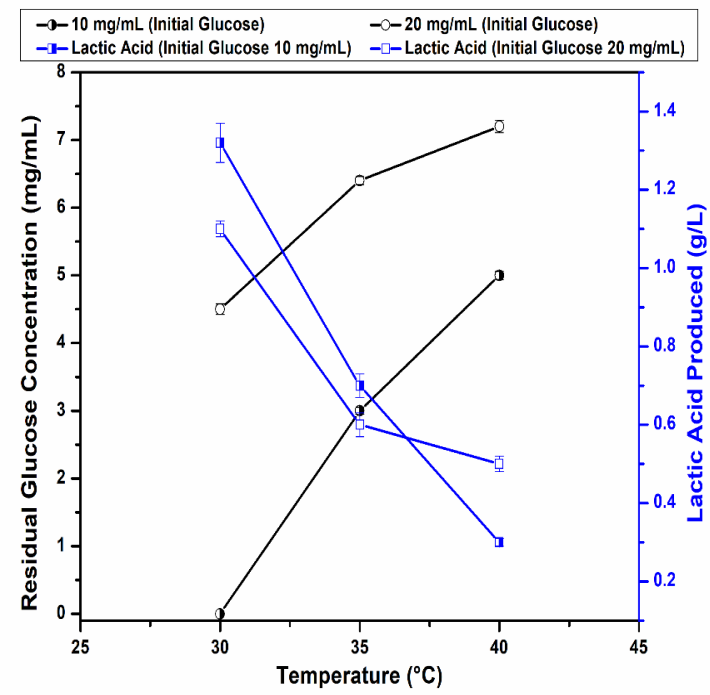

Figure 1: Effect of temperature on glucose consumption and lactic acid production

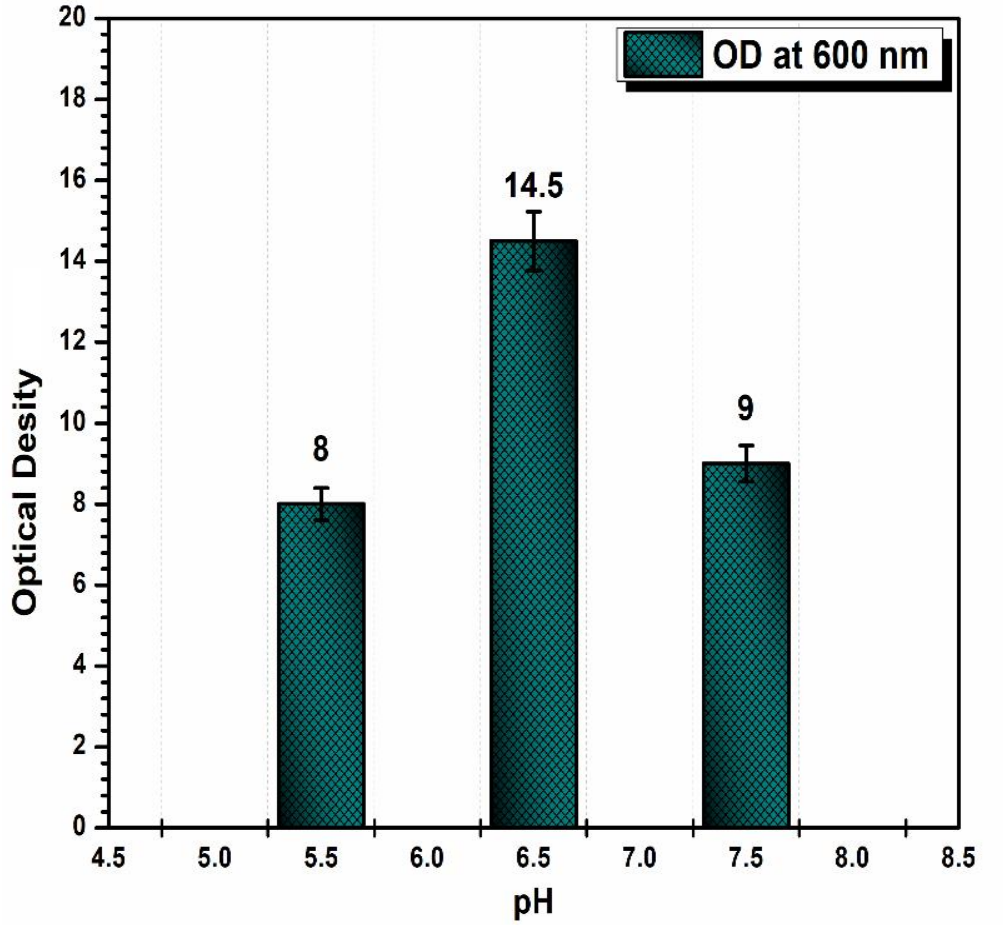

Figure: 2: Effect of pH on Optical Density at 600 nm

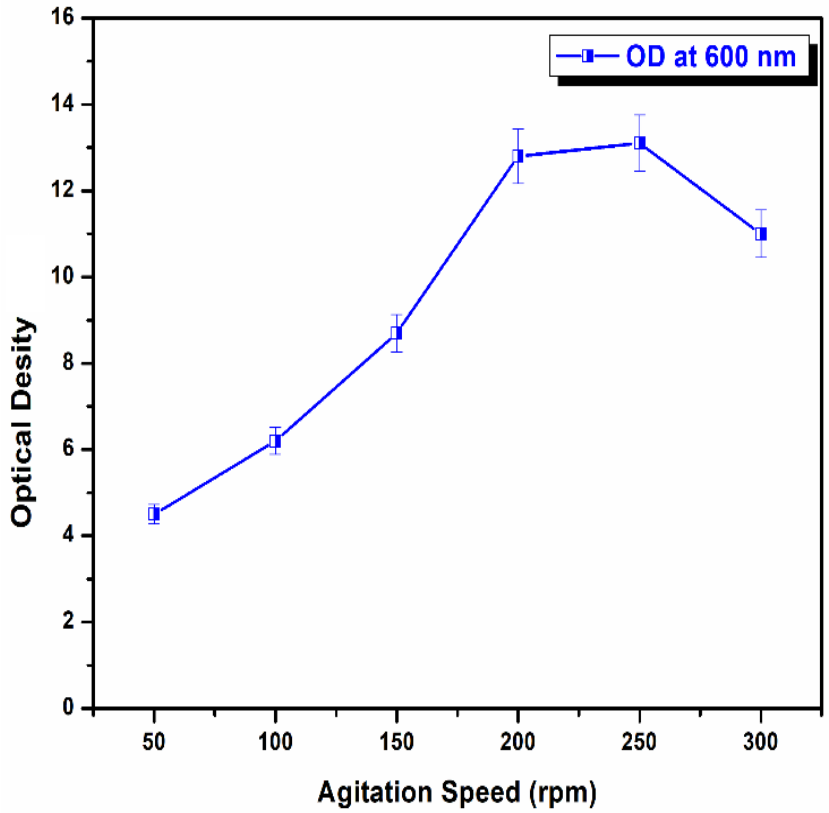

Figure 3: Effect of agitation speed on Optical Density at 600 nm

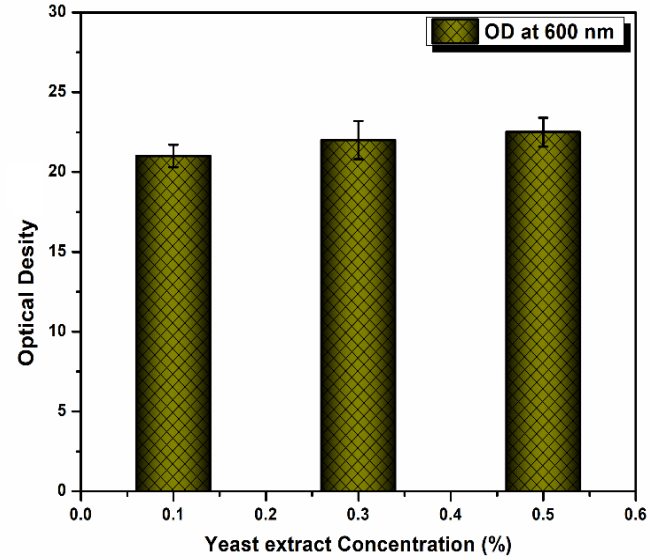

Figure 4a: Effect of Yeast Extract Concentration on Optical Density at 600 nm

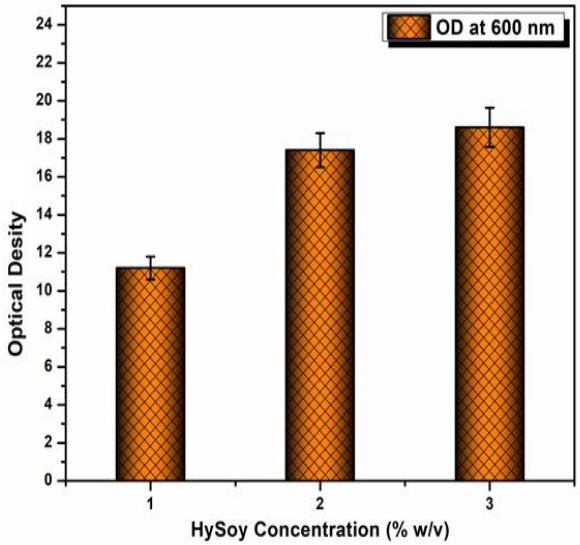

Figure 4b: Effect of HySoy on Optical Density at 600 nm

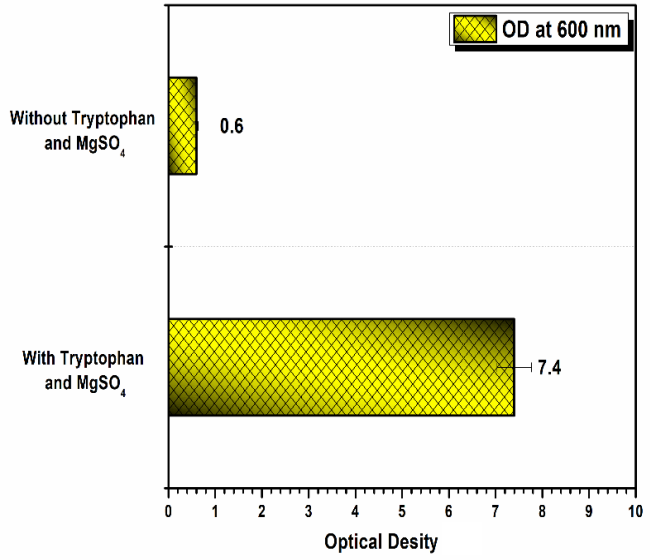

Figure 5a: Effect of Tryptophan and $MgSO_4$ on growth

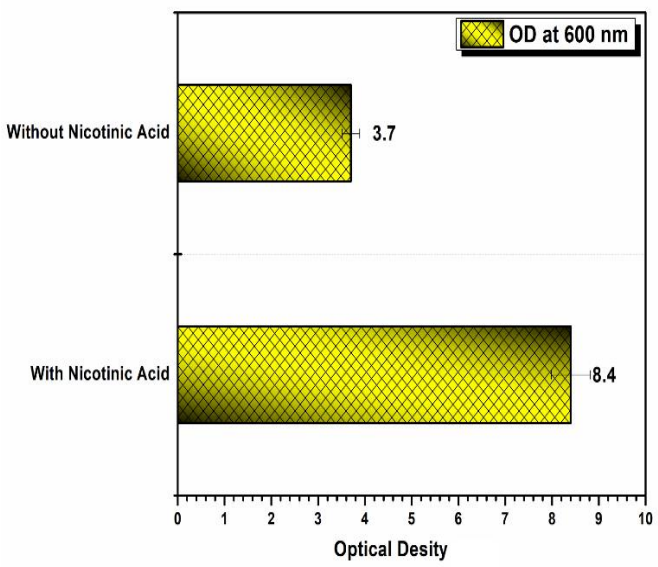

Figure 5b: Effect of Nicotinic acid on glucose consumption and growth

(ii) Effect of Nicotinic Acid on Glucose Consumption: Cultures supplemented with nicotinic acid showed increased glucose consumption compared to those without nicotinic acid. The presence of nicotinic acid correlated with enhanced bacterial growth and metabolism (Figure 5b).

Fermentation studies: The results demonstrated comparable growth at 30°C and 35°C, with a slight decline at 40°C. However, glucose consumption analysis revealed that at 30°C, a higher amount of glucose was utilized, correlating with increased polysaccharide production. This suggests that while bacterial biomass remained similar at 30°C and 35°C, metabolic activity at 30°C was more efficient for polysaccharide biosynthesis.

The observed trends align with prior studies indicating that lower temperatures often promote exopolysaccharide secretion in certain bacterial strains. Excessive heat can cause stress responses, leading to energy diversion towards survival mechanisms rather than biosynthetic pathways. Thus, maintaining a fermentation temperature of 30°C provides an optimal balance between bacterial viability and O-polysaccharide yield.

Effect of Glucose Feeding Strategy: Fed-batch fermentation exhibited significantly higher OD600 values due to sustained glucose availability compared to batch process (Figure 6).

Polysaccharide Extraction and Quantification: The efficiency of O-polysaccharide extraction was analyzed by evaluating total sugar concentration at different hydrolysis durations. The results indicate that maximum polysaccharide yield was obtained at 2 hours, beyond which degradation of the polysaccharide was observed.

Effect of Extraction Time on Polysaccharide Yield: To optimize the extraction process, polysaccharide content was measured at hourly intervals from 1 to 6 hours. The yield increased steadily up to 2 hours, after which minor fluctuations were observed, suggesting depolymerization of high-molecular-weight fractions due to prolonged acid exposure. These results indicate that hydrolysis for 2 hours at pH 5.0 and 95°C is optimal, allowing the highest polysaccharide recovery while minimizing degradation.

Molecular Weight Distribution Analysis: Size-exclusion chromatography (SEC) was performed to further characterize the extracted polysaccharide. The analysis revealed three major molecular weight peaks corresponding to different fractions of polysaccharide integrity. The reduction in high-molecular-weight polysaccharides after 2 hours suggests degradation, reinforcing the observation that prolonged hydrolysis leads to loss of structural integrity.

Discussion

This study systematically optimized the growth conditions for Salmonella enterica serovar paratyphi to enhance O-polysaccharide production. The findings provide insights into the effect of temperature, pH, agitation, nutrient supplementation and glucose feeding strategies on bacterial growth and metabolic activity.

Additionally, polysaccharide extraction was optimized to ensure maximum yield while preserving molecular integrity. The results obtained contribute to the broader field of polysaccharide-based vaccine development, where the ability to produce high-quality antigenic components is crucial.

Effect of Temperature on Bacterial Growth and Polysaccharide Production: Temperature is a key parameter affecting microbial metabolism influencing enzyme activity, nutrient uptake and biomass accumulation. In this study, bacterial growth was evaluated at 30°C, 35°C and 40°C.

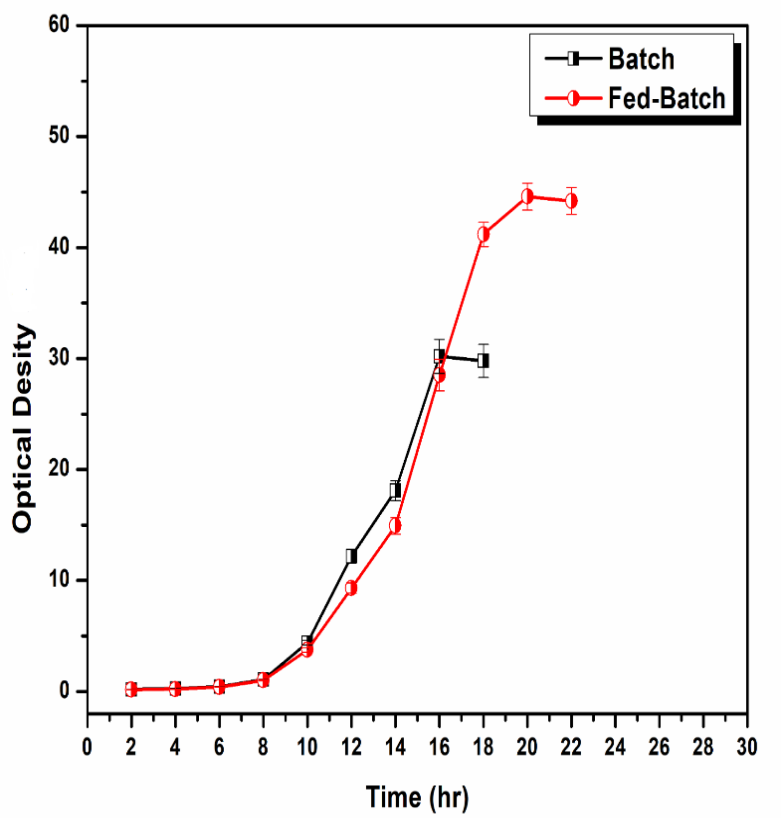

Figure 6: Growth (OD_{600}) Comparison between Batch and Fed-Batch Fermentation

Impact of pH on Growth and Polysaccharide Production: pH influences bacterial enzyme activity, membrane permeability and nutrient solubility. To assess its effect, cultures were grown at pH 5.5, 6.5 and 7.5, revealing maximum bacterial growth at pH 6.5 ± 0.3. Growth was significantly lower at pH 5.5, likely due to acid stress impairing enzymatic function and cellular homeostasis. Similarly, a higher pH of 7.5 resulted in reduced bacterial density, possibly due to ammonia toxicity or alterations in nutrient bioavailability.

Previous research supports these findings, emphasizing that neutral-to-mildly acidic pH conditions often favour optimal microbial proliferation and polysaccharide synthesis. The ability of bacteria to regulate intracellular pH is limited and deviations from the optimal range can cause impaired macromolecular stability. Thus, maintaining pH at 6.5 ensures efficient growth and enhanced O-polysaccharide accumulation.

Role of Agitation in Oxygen Transfer and Biomass Production: Agitation plays a critical role in oxygen transfer, mixing efficiency and nutrient homogeneity in bacterial cultures. The study investigated agitation rates of 150 rpm, 250 rpm and 300 rpm, finding that bacterial growth was maximal at 200–250 rpm. Lower agitation (150 rpm) resulted in poor aeration, leading to reduced biomass formation whereas excessive agitation (300 rpm) did not yield significant improvements. This suggests that while oxygen availability is necessary for growth, shear stress at higher agitation speeds may negatively impact bacterial integrity. The results are consistent with previous reports indicating that excessive mechanical agitation can damage cell membranes and alter metabolic pathways by increasing oxidative stress. Optimizing agitation speeds within the 200–250 rpm range ensures sufficient oxygen diffusion while preventing mechanical damage, contributing to enhanced cell viability and productivity.

Significance of Negative Control Studies in Growth Factor Optimization: To validate the essentiality of specific growth factors, bacterial cultures were grown with and without tryptophan, $MgSO_4$ and nicotinic acid. The results indicated significantly lower OD_{600} values in the absence of tryptophan and $MgSO_4$, confirming their necessity in protein synthesis, enzyme activation and metabolic stability. Similarly, the presence of nicotinic acid improved glucose consumption and bacterial growth, highlighting its role in redox metabolism and coenzyme synthesis.

These observations reinforce the understanding that specific micronutrients and cofactors are indispensable for bacterial survival and polysaccharide biosynthesis. The identification of critical growth-promoting components enables the development of optimized culture media, improving fermentation efficiency.

Effect of Glucose Feeding Strategies on Fermentation Efficiency: Fermentation studies compared batch and fed-batch strategies, revealing superior performance in fed-batch fermentation due to continuous glucose availability. In batch

fermentation, glucose depletion after 16 hours resulted in reduced bacterial growth, whereas fed-batch fermentation sustained higher OD600 values and polysaccharide yield by maintaining a steady-state glucose concentration. Glucose limitation is a well-known bottleneck in industrial bioprocesses, as it leads to substrate exhaustion and metabolic slowdowns. The implementation of fed-batch strategies allows controlled glucose release, preventing catabolite repression while enhancing bacterial productivity.

Polysaccharide Extraction and Hydrolysis Efficiency: The extraction of O-polysaccharide was optimized by evaluating hydrolysis duration (1–6 hours) at pH 5.0 and 95°C. The highest yield (1.853 mg/mL) was observed at 2 hours, after which degradation of high-molecular-weight polysaccharides was noted. Size-exclusion chromatography (SEC) analysis further confirmed the presence of low-molecular-weight fractions beyond 2 hours, suggesting depolymerization effects due to prolonged acid exposure.

Maintaining precise hydrolysis conditions is crucial, as excessive degradation can lead to the loss of antigenic properties in polysaccharide-based vaccines. These findings align with previous reports stating that controlled hydrolysis time and pH stabilization are critical for maximizing polysaccharide recovery while preserving molecular integrity.

Conclusion

This study successfully optimized the growth conditions for Salmonella enterica serovar Paratyphi to enhance O-polysaccharide production, a key component in polysaccharide-based vaccines. By systematically evaluating the effects of temperature, pH, agitation, nutrient supplementation and glucose feeding strategies, we established the most effective conditions for maximizing bacterial growth and metabolic activity.

The results demonstrated that 30°C was the optimal temperature for polysaccharide production, as it correlated with the highest glucose consumption and metabolic efficiency. Similarly, pH 6.5 provided the most favourable conditions for bacterial growth, while excessive acidity or alkalinity negatively impacted proliferation. Agitation studies revealed that an oxygen transfer rate of 200–250 rpm was ideal, balancing aeration and preventing shear stress. Nutrient optimization confirmed that 1.0% yeast extract and 2.0% Hysoy significantly improved biomass accumulation, whereas excess nitrogen resulted in metabolic imbalances.

Fermentation studies comparing batch and fed-batch strategies established that fed-batch fermentation outperformed batch culture due to continuous glucose availability, ensuring sustained bacterial growth and higher polysaccharide yield. Polysaccharide extraction studies identified 2 hours as the optimal hydrolysis time, beyond which degradation was observed. Size-exclusion chromatography (SEC) analysis further confirmed molecular weight reduction with prolonged hydrolysis, reinforcing the need for controlled extraction conditions.

These findings contribute to the development of scalable bioprocesses for vaccine production, optimizing polysaccharide yield while minimizing resource utilization. Future research should focus on genetic modifications to enhance bacterial productivity and alternative purification techniques to improve polysaccharide purity. Additionally, integrating machine learning-based predictive modeling could streamline fermentation control strategies, improving industrial reproducibility.

In conclusion, this study provides a comprehensive framework for large-scale O-polysaccharide production, offering valuable insights for biopharmaceutical applications and vaccine development. The optimized methodologies presented here can serve as a foundation for future advancements in microbial polysaccharide engineering and industrial biotechnology.

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